RESEARCH COMMUNICATION

Mda-9/syntenin Promotes Human Brain Glioma Migration through Focal Adhesion Kinase (FAK)-JNK and FAK-AKT Signaling

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Abstract

Invasion is usually recognized as the main reason for the high recurrence and death rates of glioma and restricts the efficacy of surgery and other therapies. Therefore, we aimed to investigate the mechanism involved in promotion effects of mda-9/syntenin on human glioma cell migration. The wound healing method was used to test the migration ability of human glioma cells CHG-5 and CHG-hS, stably overexpressing mda-9/syntenin. Western blotting was performed to determine the expression and phosphorylation of focal adhesion kinase (FAK) and JNK in CHG-5 and CHG-hS cells. The migration ability of CHG-hS cells was significantly higher than that of CHG-5 cells in fibronectin (FN)-coated culture plates. Phosphorylation of FAK on tyrosine 397, 576, and 925 sites was increased with time elapsed in CHG-hS cells. However, phosphorylated FAK on the tyrosine 861 site was not changed. Phosphorylated Src, JNK and Akt levels in CHG-hS cells were also significantly upregulated. Phosphorylation of JNK and Akt were abolished by the specific inhibitors SP600125 and LY294002, respectively. and the migration ability of CHG-hS cells was decreased, indicating that the JNK and PI3K/Akt pathways play important roles in regulating mda-9/syntenin-induced human brain glioma migration. Our results indicate Mda-9/syntenin overexpression could activate FAK-JNK and FAK-Akt signaling and then enhance the migration capacity of human brain glioma cells.

Keywords: Glioma - mda-9/syntenin - focal adhesion kinase - migration

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Introduction

Malignant gliomas, one of the most common tumors in human brain, are highly infiltrative and invasive tumors (Wen and Kesari, 2008). Invasion is usually recognized as the main reason for the high recurrence and death rates of glioma and migration of tumor cells plays an important role in the invasion of glioma and restricts the efficacy of surgery and other therapies (Nakada et al., 2007; Jing et al., 2011). Although many studies showed that many factors were involved in the invasion of glioma, including adhesion molecules, extracellular matrix (ECM), protease system and angiogenesis, the exact molecular mechanism and process of the invasive growth of glioma has not been clearly elucidated (Salhia et al., 2006; Wang et al., 2011). Therefore, there is an urgent need to elucidate the molecular mechanisms underlying migration and invasion of human glioma.

Melanoma differentiation associated gene-9 (mda-9), also called syntenin, is a scaffolding protein cloned from melanoma, containing PDZ domains with multiple biological activities (Lin et al., 1998). Previous studies have showed that mda-9/syntenin could affect the cell shape and promote the migration and invasion of many types of cancer cells, such as melanoma in which high level of mda-9/syntenin has been detected (Beekman and Coffer, 2008; Sarkar et al., 2008). Therefore, mda-9/syntenin has been considered to be a positive regulator of tumor cell progression in human melanoma and other tumors (Rondepierre et al., 2010). Mda-9/syntenin regulates cell migration and invasion through focal adhesion kinase (FAK)-dependent signaling pathways, including c-Jun N-terminal kinase (JNK) and NF-kappaB (NF-ϰB) (Boukerche et al., 2005; Boukerche et al., 2007; Hwangbo et al.). c-Src, which is a prototype of the nine-member family of structurally related Src family tyrosine kinases (SFKs), is involved in many biological processes, including increased cell motility, invasiveness, and survival (Thomas and Brugge, 1997). Upon integrin engagement, FAK together with Src form a dual-kinase complex that stimulates tumor migration and metastasis (Cary et al., 1996). Mda-9/syntenin may interacts with c-Src and lead to an increase in FAK/c-Src complex formation and c-Src activation and enhanced

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tumor cell invasion and metastatic spread (Boukerche et al., 2008). Inhibiting mda-9/syntenin with an adenovirus expressing antisense mda-9/syntenin inhibits melanoma cell migration, anchorage-independent growth, and invasion (Boukerche et al., 2007). With the knowledge above, we can reach a conclusion that upregulation and activation of mda-9/syntenin and its interacting partners are key steps for melanoma cells migration, invasion and metastasis.

In our previous study, we have shown that there was mda-9/syntenin expression in high-grade glioma. However, the relationship between the expression of mda-9/syntenin and the metastasis of glioma has not been investigated. Therefore, we performed the present study to investigate whether overexpressed mda-9/syntenin stimulated glioma cells migration and the underlying mechanisms.

Materials and Methods

Reagents

Antibodies against mda-9/syntenin (Abcam, Cambridge, MA, USA), p-FAK (Tyr 397, Tyr 576, Tyr 861, Tyr 925) (Abcam), p-Src (Biosynthesis Biothchnology, Beijing, China), p-JNK (Biosynthesis Biothchnology), p-Akt (Biosynthesis Biothchnology) and β-actin (Sigma-Aldrich, St. Louis, MO, USA) were used in this study. Fibronectin (FN), Poly-l-lysine (PL), JNK inhibitor SP600125 and PI3K inhibitor LY294002 were purchased from Sigma-Aldrich.

Construction of plasmid (pEGFP-N1-hSyntenin)

CDCBP reference sequence was used for primer designing on Primer Premier 5.0 software, and restriction sites (XhoI and BamhI) and protective bases were added to the 5' end of the forward and reverse primers respectively. The primers sequence was as follows: forward, 5'-ATT CTC GAG ATG TCT CTC TAT CCA TCT CTC G-3'; reverse, 5'-ATT GGA TCC TCA ACC TCA GGA ATG GTG TGG TC-3'. The estimated length of the product was 897 bp. Total RNA was isolated from human glioma with a RNA Extraction Kit from Promega and cDNA was synthesized with a first strand synthesis kit (TAKARA, Shanghai, China). PCR reaction was performed with a GeneAmp PCR System 9700 (ABI, Foster City, CA, USA) and started with a polymerase activation step at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 50 s, and a final extension at 72 °C for 10 min. Amplified gene with restriction sites was then cloned in mammalian expression vector pEGFP-N1 (Clontech, Palo Alto, CA, USA). Constructed pEGFP-N1-hSyntenin plasmid was confirmed through PCR, restriction digestion and sequencing.

Cell culture and transfection

Human glioma cell line CHG-5 is a gift from the institute of pathology, third military medical university. CHG-5 cell was cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 units/ml of streptomycin, at 37°C, 5% CO_2 and 95% humidity. CHG-hS, a cell line stably expressing mda-9/syntenin, was constructed by transfection of pEGFP-N1-hSyntenin plasmid with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. After 72 hours of transfection, cells were given selection with G418 initially with 400 μg/ml for selecting stable clones then after 14 days were given 200 μg/ml. The medium was changed after every 72 hours. Colonies of G418 resistant cells were selected and grown further and confirmed with immunofluorescence and western blot. pEGFP-N1 vector was transfected into the CHG-5 cells as a control (CHG-P).

Wound healing assay

Six-well plates were coated with FN or PL. CHG-hS and CHG-P cells were seeded in the coated wells and were cultured to ~80% confluence. Wound healing assay was performed as previously described (Yarrow et al., 2004). Briefly, gently and slowly scratch the monolayer with a 200 μl pipette tip across the center of the well. Scratch another straight line perpendicular to the first wound line to create a cross in each well. After scratching, gently wash the well twice with medium to remove the detached cells and add fresh medium into the wells. Cells were cultured at 37 °C for additional 10 hours. Pictures were taken at 0 and 10 hours after wounding.

Western blot

Total protein was extracted as previously described (Du et al., 2011). Briefly, the cells were lyzed with RIPA lysis buffer (Beyotime, Haimen, Jiangsu, China) and then centrifuged at 12000 g for 15 min at 4 °C. The supernatants were collected for the next western blot. Protein concentration was determined with BCA method. Protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to the Polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, US). The blot was then probed with primary antibody followed by reaction with horseradish peroxidase-conjugated secondary antibody. The signal was detected using enhanced chemiluminescence and recorded on X-ray film.

Statistical analysis

Data are expressed as means ± S.D. Statistical significance was determined using SPSS 11.0 for Windows. One-way ANOVA was performed for multiple comparisons followed by Fisher LSD post-hoc comparisons. Differences were deemed significant if $P<0.05$.

Results

Identification of mda-9/syntenin expression in CHG-hS cells

To confirm the mda-9/syntenin protein was actually highly expressed in pEGFP-N1-hSyntenin plasmid transfected CHG-5 cells, we performed western blot to detect the mda-9/syntenin expression. As shown in Figure 1, mda-9/syntenin protein was highly expressed in CHG-hS cells. But in pEGFP-N1 vector transfected CHG-P cells

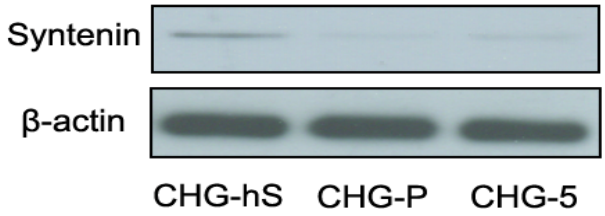

Figure 1. Expression of Syntenin in CHG-5 Cells Transfected with Syntenin Expressing Plasmid or Control Plasmid. Cells were harvested and the proteins were analyzed by western blot to detect the mda-9/syntenin expression

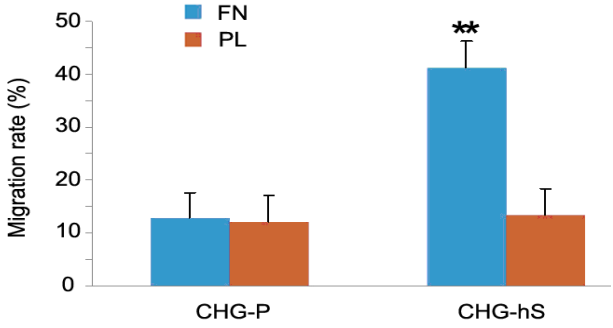

Figure 2. Migration Ability of CHG-5 Cells Transfected with Syntenin Expressing Plasmid or Control Plasmid. Cells were seeded in FN- or PL- coated plates. Wound healing assay was performed to examine the migration ability of different CHG-5 cells. Each bar represents the mean ± SD. ** P<0.01 compared with CHG-P cells

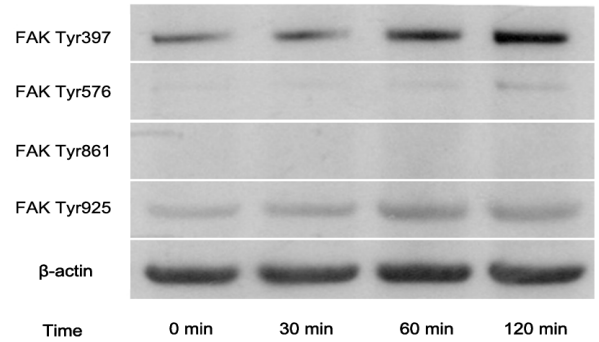

Figure 3. Phosphorylation of FAK at Different Tyrosine Residues. CHG-hS cells were stimulated with FN for indicated times and then protein was extracted. Western blot was performed to examine the phosphorylation of FAK at different tyrosine residues. β-actin was used as a loading control

and CHG-5 cells, very low expression of mda-9/syntenin protein was detected. The results indicate that mda-9/syntenin protein is stably expressed in CHG-hS cells.

Migration of CHG-hS cells in FN- or PL-coated plates In FN-coated culture plate, the migration rate of CHG-hS cells was significantly higher than that of CHG5 cells (P<0.05) (Figure 2). However, in PL-coated culture plate, no significant difference was noted between the migration rate of CHG-hS cells and CHG-5 cells, suggesting mda-9/syntenin only increases the cells migration ability in specific matrix.

Mda-9/syntenin increased FAK phosphorylation in FN-coated plate

FAK, a crucial factor in integrin-mediated signaling pathways, plays an important role in the assembly of focal

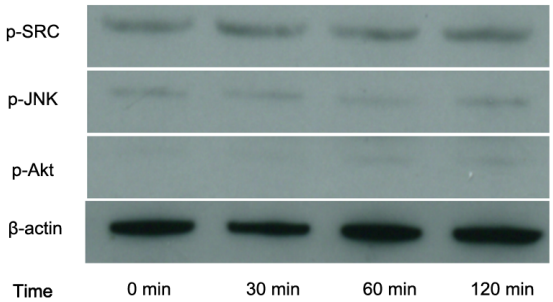

Figure 4. Phosphorylation of SRC, JNK and Akt at Different Tyrosine Residues. CHG-hS cells were stimulated with FN for indicated times and then protein was extracted. Western blot was performed to examine the phosphorylation of SRC, JNK and Akt at different tyrosine residues. β-actin was used as a loading control

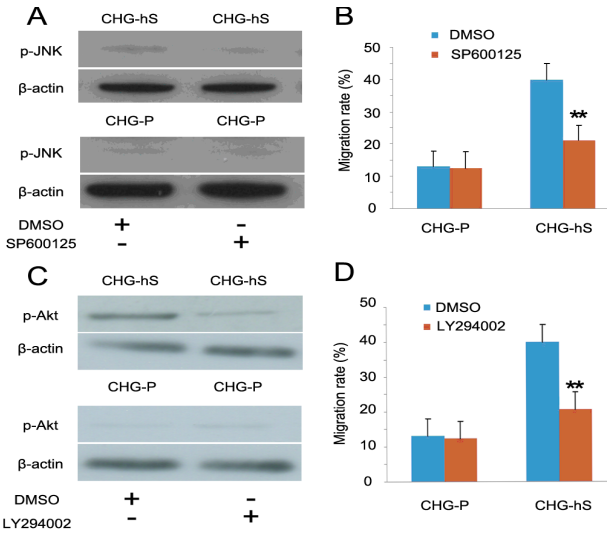

Figure 5. JNK and Akt inhibitors, SP600125 and LY294002, Decreased Mda-9/syntenin Induced Cell Migration. Upon FN stimulation, CHG-hS and CHG-P cells were incubated simultaneously with or without (A) JNK and (C) Akt inhibitors, SP600125 and LY294002 for 2 h, respectively. Then protein was extracted and western blot was performed to determine the activation of JNK and Akt. CHG-hS and CHG-P cells were also seed in FN-coated plates with or without (B) JNK and (D) Akt inhibitors. Then the cells were subjected to wound healing analysis as described above. Each bar represents the mean ± SD. ** P<0.01 compared with DMSO groups

contact structures and can influence the organization of cytoskeleton and membrane protrusions to regulate cell movement (Richardson and Parsons, 1996). Previous study showed that phosphorylation of FAK was increased significantly in melanoma cells by mda-9/syntenin (Boukerche et al., 2005). To investigate whether the enhanced migration ability of glioma cell was related to the phosphorylation of FAK, we performed western blot to examine the phosphorylation of FAK on different tyrosine residues (Figure 3). After FN stimulation for 60 and 120 minutes, phosphorylation of FAK at tyrosine 397, 576, 925 sites (p-FAK Tyr397, p-FAK Tyr576, p-FAK Tyr925) was increased significantly. However, phosphorylation of FAK at tyrosine 861 (p-FAK Tyr861) was not observed.
Mda-9/syntenin induced phosphorylation of Src, JNK and Akt

To further study the mechanisms of mda/syntenin induced cell migration in CHG-5, we detected some

molecules involved in FAK signaling. Upon FN stimulated, Src was phosphorylated in CHG-hS cells (Figure 4). Phosphorylation of JNK and Akt was also upregulated in CHG-hS cells when seeded in FN-coated culture plate.

Migration of CHG-hS cells was blocked by JNK MAPK and PI3K inhibitors

To further confirm JNK and PI3K/Akt pathways were involved in mda-9/syntenin- induced CHG-hS cell migration, specific JNK and PI3K inhibitors, SP600125 and LY294002, were used respectively. The phosphorylation of JNK and Akt was attenuated by SP600125 and LY294002 respectively (Figure 5). Subsequently, the cell migration rate was also decreased by SP600125 and LY294002.

Discussion

In the present study, we investigated the effects of mda-9/syntenin on human brain glioma cells migration. First, we constructed an expression plasmid of mda-9/syntenin and stably transfected into CHG-5 cells. The expression of mda-9/syntenin was confirmed by western blot. In FN-coated culture plate, mda-9/syntenin could enhance the migration ability of CHG-5 cell. To elucidating the mechanisms of mda-9/syntenin's effect, we tested some molecules involved in FAK signaling. Mda-9/syntenin could upregulate the phosphorylation of FAK at tyrosine 397, 576, 925 sites. Mda-9/syntenin also increased the phosphorylation of Src, JNK and Akt. Our results indicate mda-9/syntenin may promote glioma migration through FAK/JNK and FAK/Akt pathways.

Cell adhesion and migration is an important step of tumor metastasis. When the cells contact with the extracellular matrix (ECM), extended filopodia binds to ECM through integrins and mediates the formation of focal adhesion, which is a power source of cell migration (Meurice et al., 2010). Mda-9/syntenin could remodel the actin cytoskeleton, induce the formation of a variety of plasma membrane structures including goffer, lamellipodia and neurite-like structure in neurons and different types of tumor cells, indicating it may play a role in tumor cell adhesion and migration (Zimmermann et al., 2001; Hirbec et al., 2005; Sulka et al., 2009). Some clinical trials have confirmed that mda-9/syntenin was overexpressed in melanoma, metastatic breast cancer and gastrointestinal tumors and the level of mda-9/syntenin was closely correlated with the tumor malignant grades and tumor metastasis (Koo et al., 2002; Helmke et al., 2004; Boukerche et al., 2005; Boukerche et al., 2008). In the present study, we found that the migration ability of CHG-hS, but not CHG-5 was elevated in FN-coated culture plates. However, in PL-coated culture plates, the migration ability of CHG-hS and CHG-5 was nearly the same. This different results from FN- and PL-coated plates may be due to FN, a main component of the ECM which could promote melanoma invasion, can induce the mda-9/syntenin expression (Hwangbo et al., 2010), but PL may not stimulate the expression of mda-9/syntenin.

FAK, a tyrosine kinase is activated in response to integrin-matrix interactions. It has been implicated that FAK plays a central role in the regulation of a multitude of biological responses including cell proliferation, survival, and migration (Mitra et al., 2005). Increased FAK expression correlated with enhanced invasiveness and motility of human tumor cells (Akasaka et al., 1995; Owens et al., 1995). It has been established that interaction of mda-9/syntenin with c-Src promotes the formation of an active FAK/c-Src signaling complex, leading to enhanced tumor cell invasion and metastatic spread in human melanoma (Boukerche et al., 2008; Boukerche et al., 2010). In the present study, we showed that mda-9/syntenin expression in glioma cells significantly increased the p-FAK and p-Src in FN-coated plates, suggesting the activation of FAK/c-Src signaling is also a crucial step for mda-9/syntenin-induced human glioma migration.

Boukerche et al previously documented that forced mda-9/syntenin expression triggered a signaling cascade resulting in the phosphorylation of FAK and activation of JNK and p38 MAPK in human melanoma cells, leading to secretion of matrix metalloproteinase (MMP)-2 (Boukerche et al., 2005; Boukerche et al., 2007). In accordance with previous results, we found that JNK was activated in CHG-hS cells. However, p-Akt, which had little change in human melanoma cells with mda-9/syntenin expression, was also increased in CHG-hS cells. With JNK inhibitor or Akt inhibitor treatment, the phosphorylation of JNK and Akt was blocked and the migration ability of CHG-hS cells was decreased. Our results indicate that JNK and PI3K/Akt are involved in the CHG-hS cells migration and are downstream molecules of FAK/c-Src signaling in CHG-hS cells.

In conclusion, our results suggest that mda-9/syntenin expression promotes the migration of human brain glioma cells through the activation of Src/FAK/JNK and Src/FAK/PI3K/Akt signaling pathways. Our study provides a new view of the molecular mechanisms of glioma cell migration and invasion. Mda-9/syntenin may serve as a potential therapeutic target for the treatment of human glioma metastasis.

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