主論文の要旨

Girdin/GIV Regulates Collective Cancer Cell Migration by Controlling Cell Adhesion and Cytoskeletal Organization

（Girdin/GIVは細胞間接着と細胞骨格の制御を介して癌細胞の集団的移動を制御する）

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Introduction

In this study, we studied the role of the actin-binding protein Girdin, a specific regulator of collective migration of neuroblasts in the brain, in collective cancer cell migration. We and others have previously shown that Girdin is expressed by multiple types of cancers, where its expression correlated with cancer progression. In this study, we showed a role of Girdin in the collective invasion of skin cancer cells, where it interacts with β-catenin, a component of the E-cadherin complex. Our data showed that Girdin is indispensable for stable cell-cell interaction, supracellular cytoskeletal organization, and the collective migration of cancer. Finally, we also examined the clinical relevance of Girdin expression in the progression of human skin cancer.

Materials and methods

The human cancer cell lines A431,DLD1 and HeLa, the Madin-Darby canine kidney epithelial cell line MDCK and the human embryonic kidney epithelial cell line 293FT were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cDNAs for human β-catenin and α-catenin were generously provided by Frank Costantini (Columbia University, NY, USA) and Takashi Watanabe (Nagoya University, Nagoya, Japan), respectively. For cell dissociation assay, We followed a standard protocol previously described that measures the strength of cell-cell adhesion. The isolation of nuclear, membrane and cytoskeletal fractions of A431 cells was done using an S-PEK cell fractionation kit (539790, Merck).For organotypic model, immortalized CAF derived from a human vulvar cancer (5×10^5) were embedded in a mixture of type I collagen and Matrigel (BD Biosciences), yielding a final collagen concentration of 4 mg/mL and a final Matrigel concentration of 2 mg/mL. We followed a standard protocol for immunohistochemical staining of mouse brain and human skin cancer tissues.

Results

We first conducted experiments to visualize collective migration in vitro. We reproducibly observed that the behavior of A431 single cells and cell groups on plastic dishes was significantly different from that on collagen gels (**Figures 1A, B**). We next depleted Girdin with shRNA to determine the impact on the collective behavior of A431 cells seeded on collagen gels (**Figures 1C-G**).These data suggested that Girdin plays an essential role in collective cancer cell migration.

Immunoprecipitation (IP) experiments showed that β-catenin was co- immunoprecipitated by Girdin antibody in several types of cancer cells or immortalized epithelial cell A431(**Figures 2A**). α-catenin was also co-immunoprecipitated with Girdin, leading to the speculation that Girdin, α-catenin, β-catenin, and E-cadherin might form a complex in

these cells. The interaction was also confirmed by a reciprocal IP test that showed that β-catenin immunoprecipitates contained Girdin, α-catenin, and E-cadherin in A431 cells (**Figure 2B**). Immunofluorescent staining on A431 and MDCK cells clearly showed the colocalization of Girdin and β- and α-catenins at cell-cell adhesion sites on either plastic dishes or collagen gels, suggesting that Girdin plays a role in the regulation of intercellular adhesion (**Figures 2C, D**). These data suggested that Girdin/β-catenin interaction may be vital for cell motility.

To address which of Girdin's domains is responsible for β-catenin interaction, we expressed the domains of Girdin and β-catenin in 293FT cells, and mapped interacting domains by IP tests (**Figures 2F, G**). The data showed that Girdin's CT domain interacts with the N-terminal domain of β-catenin. An *in vitro* binding assay using purified recombinant GST-fused β-catenin and the His-fused Girdin CT domain showed their direct interaction (**Figures 2H, I**).

We found that Girdin localization at cell-cell contact sites was more prominent in high density cultured A431 cells that formed mature cell-cell contacts than in low density cultured cells with immature contacts (**Figure 3A**). We used siRNA to deplete the endogenous levels of Girdin in HeLa cells. This treatment induced HeLa cells to scatter such that the cells could not adhere to each other tightly or regularly (**Figures 3B, C**). These data indicated that Girdin played an essential role in cell-cell adhesion. We next examined the role of Girdin in calcium-dependent, E-cadherin-mediated adhesion between cells as determined by a cell dissociation assay (**Figures 3D-H**). The data suggested the involvement of Girdin in E-cadherin-mediated cell-cell adhesion.

A fractionation experiment showed that the amounts of β- and α-catenins and E-cadherin that reside in the cytoskeletal fraction were decreased, whereas those in a membrane fraction were increased by Girdin depletion, suggesting a role of Girdin in cytoskeletal association of the catenin protein complex (**Figures 4A, B**). Another difference between control and Girdin-depleted cells was revealed by the staining of the actin filaments by phalloidin (**Figure 4C**). Given the importance of supracellular cytoskeletal organization, we speculate that Girdin regulates collective migration by facilitating the cytoskeletal association of β-catenin and related cytoskeletal reorganization (**Figure 4D**). Furthermore, the overexpression of the NT domain of β-catenin, which is responsible for binding with Girdin, led to a competitive disruption of endogenous Girdin/β-catenin interaction (**Figure 5A**) and directional migration of A431 cell groups on collagen gels (**Figures 5B-D**) supporting the physiological role of Girdin/β-catenin interaction in collective cell migration.

Our data on A431 cells cultured on collagen gels suggested the importance of Girdin in their collective invasion. To support this hypothesis, we adopted an organotypic culture model in which we prepared Matrigel containing cancer-associated fibroblasts (CAFs) on

which we seeded A431 cells, rendering them air-exposed (**Figure 6A**). We found that the depletion of Girdin, as well as that of the other components of the E-cadherin/catenin complex, attenuated the number of collective cell groups (clusters) invading into Matrigel, but not cell number in each group, further confirming a role of Girdin in collective invasion of cancer cells (**Figures 6B, C**). The depth of invasion was regulated by Girdin and E-cadherin, but not β- and α-catenins (**Figure 6D**), suggesting that Girdin and E-cadherin promote collective invasion with high persistency, but not elucidating the role of the E-cadherin protein complex.

Finally, we investigated the significance of Girdin expression in the progression of squamous cell carcinoma of the skin that is known to collectively invade the stroma. To do this, we developed a scoring system by which the expression levels were evaluated by both intensity scores (IS) and proportion scores (PS) (**Figure 6E**). Statistical analysis of 76 cases of skin cancer showed that the expression level of Girdin was upregulated in most cases of squamous cell carcinoma of the skin (**Figure 6F**).

Discussion

One should consider a previous study that showed retrograde flow of N-cadherin along the cell-cell contact sites during collective migration of primary cultured astrocytes.[15] Our observation using A431 cells expressing E-cadherin-Ruby, however, did not reveal any significant movement along the cell-cell junctions or intracellular trafficking of E-cadherin during collective migration. Those results suggest that the mechanism underlying cadherin dynamics in cancer cells might be distinct from that in neural cells. Further studies are needed to clarify the cell- or tissue-specific mechanisms for collective migration.

Conclusion

We showed that Girdin is involved in the function of the E-cadherin/catenin complex, where it controls the stability of cell-cell adhesion, and at the same time regulates supracellular cytoskeletal organization.