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Cell Surface Protein C23 Affects EGF-EGFR Induced Activation of ERK and PI3K-AKT Pathways

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Abstract The epidermal growth factor (EGF) pathway has been reported as canonical causes in cancer development. Meanwhile, the involvement of C23 in multiple signaling pathways has been also investigated (Lv et al., 2014). However, the effect of C23 on EGF pathway in glioblastoma is not fully characterized. In the present study, C23 and the epidermal growth factor receptor (EGFR) of U251 cell line were inhibited by C23 and EGFR antibodies, respectively; and then C23 and EGFR siRNAs were used to knock down endogenous C23 and EGFR, respectively. In addition, soft-agar and MTT assay were also introduced. Compared with control, either C23 or EGFR antibodies efficiently repressed the phosphorylation levels of ERK1/2 ($p<0.000$) and AKT ($p<0.000$). Similarly, either C23 or EGFR siRNAs indeed resulted in C23 and EGFR knockdown, and further suppressed the expression of p-ERK1/2 and p-AKT. Most importantly, immunoprecipitation revealed C23 interacted with EGFR once U251 was exposed to EGF treatment. In addition, the MTT and soft-agar assay also identified that C23 or EGFR siRNAs could obviously affected cell growth ($p=0.004$) and invasiveness, as cell viability and colony formation decreased markedly. Our results suggest that C23 plays a crucial role in activation of EGF-induced ERK and PI3K-AKT pathways via interacting with EGFR; furthermore, C23 could be indicative of an important factor in glioblastoma development and a useful target for glioblastoma treatment.

Keywords C23 · EGF · ERK · PI3K-AKT · Glioblastoma

Introduction

It has been reported that glioblastoma is the most common one of human brain tumors (Lawton et al. 2012; Wakabayashi 2011), and a thorough understanding of the molecular mechanisms will facilitate the identification of markers. EGF pathway acts as a conventional but important target, and has been considered a therapeutic target, due to its involvement in cell proliferation (Fan et al. 2009; Paugh et al. 2008). EGF can bind with high affinity to EGFR on the cell surface and stimulate the intrinsic protein-tyrosine kinase activity of the receptor. EGF pathway mainly included the RAS/RAF/MEK/ERK and PI3K/AKT pathways (Dai et al. 2013; Dent 2014). Both pathways operate as a complex network and also provide the rationale for developing therapeutic agents.

On the other hand, C23 has been reported as a kind of nuclear proteins, which is situated on the nucleolus and cytoplasm (González et al. 2009; Li et al. 1996). In particular, C23 is also determined on the cell surface. Till now, it has been reported that cell surface-expressed C23 on THP-1 monocytes was involved in the inflammatory response induced by lipopolysaccharide (LPS) (Wang et al. 2011), and was suggestive of a receptor of laminin-1 (Turck et al. 2006). In addition, altered C23 expression has been investigated in colon cancer (Wu et al. 2014), gastric cancer (Qiu et al. 2013), and

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gliomas (Galzio et al. 2012), et al. The recent reports showed that the nucleolin-FGF1 interaction was required for the intranuclear phosphorylation of FGF1 (Sletten et al. 2014). And, C23 can also affect the tumor growth and angiogenesis (Destouches et al. 2008). Thus, it is plausible that cell surface C23 might be associated with EGF pathway.

Here, we investigated the effect of C23 in EGF signaling pathway using glioblastoma cell line U251. C23 and EGFR siRNAs as well as antibodies were introduced to knock down and block C23 of U251 plasma membrane, and then ERK1/2 and AKT proteins were detected using Western blot. Besides, we identified the relationship between C23 and EGFR by immunoprecipitation.

Materials and Methods

Cell Culture, Treatment, and siRNA Transfection

The human glioma cell lines U251 (American Type Culture Collection) was used in this study. U251 cell line was grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10 % fetal bovine serum (FBS; Hyclone) and maintained at 37 °C in an atmosphere of 5 % CO2 and 95 % room air. The cells were grown to 60 % confluence, followed by serum starvation for 16 h. As the siRNA protocol told, the cells were transfected with human si-C23, si-EGFR, or negative control siRNA (si-control) (Santa Cruz) at a final concentration of 100 nM in the presence of an Oligofectamine reagent (Invitrogen, Carlsbad, CA), as the manufacturer's protocols. Following transfection, the cells were harvested at 72 h for protein extraction and additional analysis. All experiments were conducted in triplicate, and representative results are reported.

Western Blot Analysis

Cells were treated with PBS, and then 1 % of Triton X-100 lysis buffer (20 mM Tris-HCl, pH 7.4, 10 mM EGTA, 10 mM MgCl2, 1 mM benzamidine, 60 mM β-glycerophosphate, 1 mM Na3VO4, 20 mM NaF, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride) was added. Then the supernatants were collected by centrifuge. Protein concentration was tested using the protein assay BCA kit, and then equal amounts of proteins were loaded on SDS-PAGE gels. Subsequently, proteins were transferred to PVDF membranes (Millipore, Beaford, MA) and were immunoblotted with antibodies (1:1000): antibodies: anti-p-ERK1/2, anti-ERK1/2, anti-p-AKT, anti-AKT, anti-EGFR and anti-C23 antibodies (Santa Cruz Biotech), and anti-β-tubulin (Santa Cruz Biotech). Immunoreactive bands were visualized by chemiluminescence (ECL; Pierce) using a secondary antibodies (1:8000).

Membrane Protein Isolation and Immunoprecipitation

Membrane proteins were extracted and concentrated with Mem-PER® eukaryote membrane protein extraction kit and Pierce® SDS-PAGE Sample Prep Kit (Pierce, Rockford, USA). Membrane proteins were then incubated at 4 °C for 2 h with EGFR or C23 antibody (1:1000) (Santa Cruz Biotech). The immune complexes were isolated by stirring the mixture at 4 °C overnight with Protein A/G Plus-Agarose. And then, the samples were loaded onto 10 % SDS-PAGE for Western blotting as described by Lv et al. (2014). The C23 or EGFR antibody (1:1000) was used to detect the C23 or EGFR expression of the immune complexes, respectively.

Soft Agar Assay

A 1.5-ml layer of 0.5 % agar (wt/vol) in DMEM with 10 % FBS was poured in 35-mm Petri dishes. U251 cells were treated with control, si-C23 or si-EGFR for 24 h at 100 nM in the presence of Oligofectamine reagent (Invitrogen) and resuspended in 0.35 % agar (wt/vol) in DMEM with 10 % FBS at a density of 5,000 cells/1.5 ml; 1.5 ml of cell suspensions were poured on the top of the base layer, allowed to solidify, and incubated at 37 °C in the presence of 5 % CO2 for 21 days. The colonies were stained with 0.005 % crystal violet for 1 h. Colonies containing >10 cells were counted under an Olympus microscope (Olympus).

MTT Proliferation Assay

The effect on cellular proliferation was measured by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, based on the ability of live cells to utilize thiazolyl blue and convert it into dark blue formazan. Exponentially, growing cells were seeded into a 96-well plate at 2,000 cells per well. After treatment, cells were treated with MTT (5 g/L, Sigma) for 4 h at 37 °C, and then dimethyl sulfoxide (DMSO) was added into each well for 30 min. A 96-well microtitre plate reader (Pharmacia) was used to determine A590. All experiments were performed three times in triplicate.

Statistical Analysis

The $p<0.01$ was regarded as with statistical significance. The semi-quantitative analysis was based on Image-Pro Plus. The SPSS 13.0 statistical analysis software was used, while the

analysis of variance was employed. Data from three independent experiments was expressed as the mean±SD.

Results

ERK and PI3K-AKT Pathways were Suppressed by either C23 or EGFR Antibody

To determine the mechanism through which cell surface C23 regulates the EGF pathway, we first assessed how C23 affected the activation of ERK and PI3K-AKT pathways, we used either C23 or EGFR antibody to block U251 cells, and then ERK1/2 and AKT were analyzed. Blocking of C23 on the surface decreased the amount of the phosphorylated ERK1/2 (p-ERK1/2), at the same time, did affect the phosphorylated AKT (p-AKT) level. Conversely, the control group (EGF and IgG-con) revealed that the phosphorylated ERK1/2 and AKT were significantly upregulated following stimulation ($p<0.000$, $p<0.000$, respectively; Fig. 1a, b). These results suggested that the activation of ERK and PI3K-AKT pathways were regulated by cell surface C23. Next, we performed EGFR blocking with EGFR antibody, and observed that EGFR antibody also obviously decreased the expression of p-ERK1/2 and p-AKT, compared with control ($p<0.000$, $p<0.000$, respectively; Fig. 1a, b). All of these suggested that inhibition of C23 interfered with the ERK and PI3K-AKT pathways.

ERK and PI3K-AKT Pathways were also Suppressed by either C23 or EGFR siRNA

Because intracellular C23 and EGFR proteins were still present in U251 cells, we efficiently inhibited the expression of C23 using either C23 or EGFR siRNA. Subsequently, ERK1/2 and AKT proteins were analyzed. We observed that either C23 or EGFR was efficiently knocked down with the treatment of C23 or EGFR siRNA, respectively (Fig. 1c).

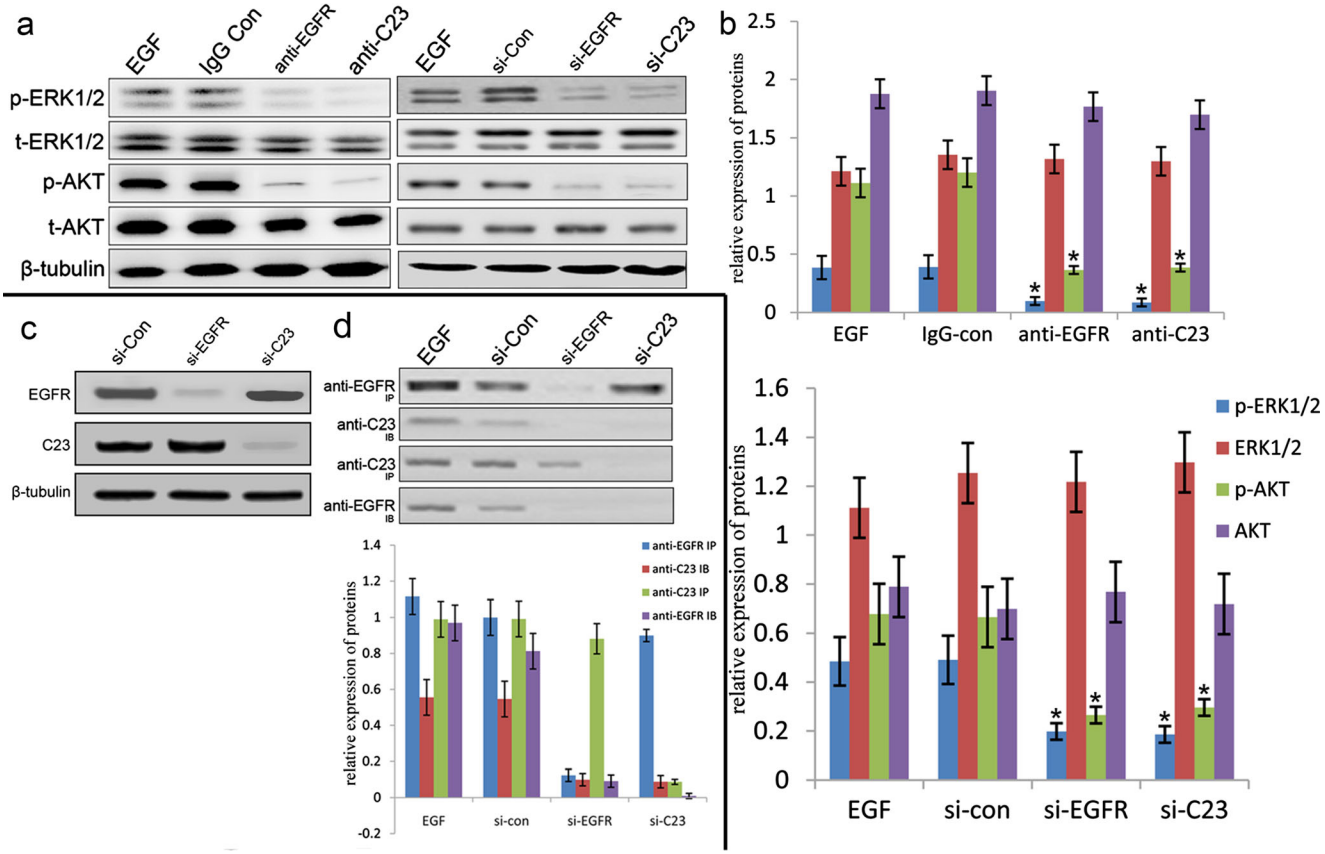

Fig. 1 Identification of C23 as a regulator of the EGF signaling pathway. U251 was serum-starved for 16 h, and then treated by anti-C23, anti-EGFR or IgG antibodies (control) as well as transfection with si-C23, si-EGFR or si-control for 72 h. Subsequently, U251 was treated with EGF (50 ng/ml) for 60 min. **a** Immunoblot in U251 cells showed the inhibitory impact of C23 and EGFR antibodies on p-ERK1/2 and p-AKT. **b** The semi-quantitative analysis showed C23 and EGFR antibodies or siRNAs efficiently suppressed the expression of p-ERK1/2 and p-AKT. The band intensities are also shown from three independent experiments. β-tubulin acted as a internal control *$p<0.000$; vs control; using Student's *t* test. **c** Either C23 or EGFR was indeed knocked down with the treatment of C23 or EGFR siRNA, respectively, compared with si-control. **d** Immunoprecipitation was conducted at 30 min following the treatment. The membrane proteins were separated as described above, and then subjected to immunoprecipitation with anti-C23 and immunoblotted with anti- EGFR, or vice versa. C23 and EGFR siRNAs prohibited the interaction between C23 and EGFR. All data from three independent experiments was expressed as the mean±SD

Not only did C23 knockdown obviously decrease the amount of p-ERK1/2, but also affected the p-AKT level. Conversely, the control group (EGF alone, and si-con) revealed that the phosphorylated ERK1/2 and AKT were not affected (Fig. 1a, b). Our results suggested that C23 really regulated the activation of ERK and PI3K-AKT pathways. Next, we conducted EGFR knockdown with EGFR siRNA, and observed that the expression level of p-ERK1/2 and p-AKT also obviously decreased with the treatment of EGFR siRNA ($p<0.000$, $p<0.000$, respectively; Fig.1b). These results indeed identified that C23 inhibited the ERK and PI3K-AKT pathways.

C23 was Identified to Interact with EGFR

Membrane proteins were immunoprecipitated with anti-C23 antibody and immunoblotted with anti-EGFR antibody, or vice versa. As shown in Fig.1d, 30 min following EGF alone treatment, EGFR was immunoprecipitated by C23 antibody in the control, whereas in the group with si-C23 or si-EGFR, C23 or EGFR was not examined in immunoprecipitates. Our data indicated that once U251 was stimulated by EGF, C23 wound bind to EGFR on plasma membrane, and then activate EGF/EGFR interaction. Taken together, C23 indeed acted as a key component in the activation of EGF pathway, further affected the ERK and PI3K-AKT pathways.

Either C23 or EGFR siRNA Affected U251 Cell Viability and Invasiveness Induced by EGF

All the above has identified the role of C23 in the activation of ERK and PI3K-AKT pathways mediated by EGF; to further characterize the effect of C23 or EGFR siRNA on U251 cell viability and invasiveness, the MTT assay and soft-agar assay were conducted. As was shown in Fig. 2, C23 or EGFR knockdown could also inhibit the viability of U251 cells; on the contrary, the control group was not affected. Differences were significant (*$p=0.004$, vs control). As expected, the soft-agar assay revealed that C23 or EGFR siRNAs effectively inhibited the invasiveness, with colony formation reduced by about 62 and 56 %. All the above indicated that C23 indeed affected cell growth and invasiveness of U251.

Discussion

Previously, EGF was identified to participate in oncogenesis via EGF pathway (ERK and PI3K-AKT pathways), especially in advanced cancers such as glioblastoma (Xie et al. 2014). At the same time, C23 has been reported to be present in kinds of cancers and affects some pathological processes. Notably, there is no evidence that C23 plays an important role in EGF signaling of glioblastoma. A thorough understanding of the molecular mechanisms will allow for the discovery of new

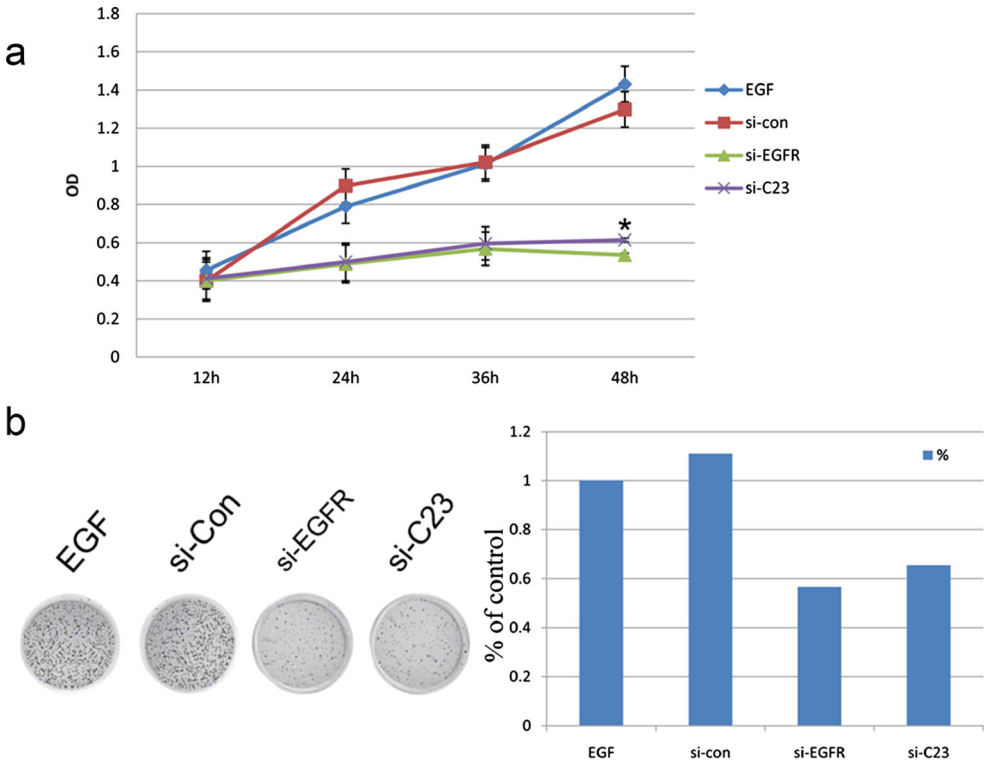

Fig. 2 The regulatory effect of C23 on U251 viability and invasiveness. Cells were transfected with si-C23, si-EGFR, or si-control for 72 h prior to exposure with EGF (50 ng/ml). **a** Cell viability was assayed by measuring MTT. Cell growth curve were plotted according to absorbance at 590 nm. *$p=0.004$, vs control; using analysis of variance of repeated measurement. Data from three independent experiments was expressed as the mean±SD. **b** The soft agar assay was performed as mentioned above. C23 and EGFR siRNAs obviously affected the colony formation

therapeutic targets (Agnihotri et al. 2013). Here, we used the glioblastoma cell line U251 as the model to investigate the association between C23 and EGF pathway. C23 and EGFR siRNAs as well as antibodies were introduced to knock down and block C23 on U251 cell surface, and then ERK1/2 and AKT proteins were immunoblotted. Furthermore, we identified the relationship between C23 and EGFR by immunoprecipitation.

Our study found that both C23 and EGFR antibodies could efficiently inhibit the EGF pathways activation. First, blocking of C23 on the surface decreased the amount of p-ERK1/2, at the same time, did affect the p-AKT level. In contrast, the IgG control revealed that p-ERK1/2 and p-AKT were upregulated. Simultaneously, we also obtained the same results by blocking EGFR on the U251 cell surface. Because EGF is highly active and is a well-known oncogenic factor in glioblastoma, these results indicated that C23 could induce oncogenesis in glioblastoma owing to its involvement in activation of the EGF pathway.

We further tested the activation of ERK and PI3K pathway to judge whether EGF pathway was inhibited by C23 inhibition. To eliminate the potential impact of endogenous C23 and EGFR in U251 cells, we knocked down the expression of C23 and EGFR using either C23 or EGFR siRNA. We observed C23 or EGFR siRNA potently decreased the amount of p-ERK1/2 and also affected the expression of p-AKT. Conversely, the control group (EGF and si-con) revealed that the p-ERK1/2 and p-AKT were not affected. Likewise, these results further suggested that C23 really regulated the activation of ERK and PI3K-AKT pathways.

In our study, the requirement of both C23 and EGFR for EGF pathways was also identified using immunoprecipitation. We demonstrated that C23 indeed interacted with EGFR. Thus, C23 more likely acted as a key component in more receptors signaling pathways. However, the underlying mechanism of the interaction and regulation between the C23 and EGFR warrants further study. In view of the involvement of ERK and PI3K-AKT pathways in cell proliferation and invasiveness (Qiu et al. 2014; Wang et al. 2014; Tsubaki et al. 2012), in the present study, the viability and invasiveness of U251 cell lines were investigated using MTT and soft agar assay. We were convinced that C23 or EGFR siRNAs reduced the cell growth and colony formation, suggesting that U251 cell proliferation and invasiveness were affected. Therefore, C23 plays an active role in EGF signaling.

As reported, Schokoroy S suggested that targeting both nucleolin and Ras may represent an additional avenue for inhibiting cancers driven by these oncogenes (Schokoroy et al. 2013). On the other hand, nucleolin was also implicated in the regulation of human astrocytoma proliferation in vitro and tumorigenicity in vivo, and may represent a potential novel therapeutic target for astrocytomas (Xu et al. 2012). According to our results, C23 maybe be a kind of tumor-causing mechanism. In addition, HB-19 pseudopeptide, a C23 antagonist that binds GAR domain, can efficiently antagonize cell surface C23, which may be recommended as regimen of glioblastoma (Watanabe et al. 2010). As mentioned above, C23 should be suggested as an efficient target for clinical treatment.

In conclusion, this study is the first to demonstrate that C23 can interact with EGFR to regulate the activation of the ERK and PI3K-AKT pathways in glioblastoma. At the same time, an interesting suggestion raised by this study is that C23 may bind to most growth factor receptors in many kinds of cancers and affect cell development via receptor signaling, which will make C23 an attractive candidate for cancer diagnosis and treatment.

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Conflict of Interest The authors state that there are no conflicts of interest to disclose.

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