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Original Article

Synergistic anticancer effect of Grb2 and ITGA1 on cancer cells highly expressing Grb2 through suppressing ERK phosphorylation

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Abstract: Background: Growth factor receptor bound protein 2 (Grb2) is known to be an adaptor protein that provides a critical link between cell surface growth factor receptors and the MAPK signaling. It was shown that high expression of Grb2 enhances cancer cells proliferation, invasion and malignant transformation. Objective: In this study, we aimed to systemically understand the function of Grb2 in cancer. Methods: The expression of Grb2 in different cancer cell lines was examined from a publicly available database and we chose two cancer cell lines highly expressing Grb2 to investigate the role of Grb2. To systemically understand the function of Grb2 in cancer cells, proteomic profiles also were analyzed. Result: The results suggested that downregulation of Grb2 reduced cell proliferation in Hela cells and Jurkat cells. In addition, knockdown of Grb2 reduced the expression of ITGA1 and inhibited the phosphorylation of ERK. Intriguingly, simultaneous inhibition of Grb2 and ITGA1 resulted in a greater inhibition of phosphorylated ERK than either inhibition of Grb2 or ITGA1, and thus triggered marked apoptosis in Hela cells and Jurkat cells. These results suggest a synergistic anticancer effect of Grb2 and ITGA1 mediated by the ERK pathway in cancer cells highly expressing Grb2. In conclusion, we provided evidence that inhibition of Grb2 and ITGA1 might be an attractive target for therapeutic intervention against the cancer growth of cancers with high Grb2 expression.

Keywords: Grb2, ITGA1, ERK, cancer

Introduction

The growth factor receptor bound protein 2 (Grb2), a ubiquitous adapter protein, mainly mediates phosphatidylinositol 3-kinase (PI3K) and extracellular signal regulated kinase (ERK) pathway [1, 2]. It is well known that high expression of Grb2 can promote the activation of signaling pathways and malignant transformation via ERK pathway. It is reflected that Grb2 plays the vital role in regulation of proliferation, invasion and malignant transformation [3, 4]. For example, amplification of Grb2 promotes breast tumor growth [5, 6]. Expression level of Grb2 is correlated with metastasis and invasion in esophageal squamous carcinoma [7, 8]. Interestingly, the functions of ITGA1 (integrin, α1) also play a critical role in above aspects. It has been reported that integrins are involved in various cancers [9].

Integrins are transmembrane receptors composed of two subunits, α and β chains. They comprise a large family of cell surface receptors, with more than 18α subunit and 8β subunit isoforms identified in mammals, provide dynamic cell to cell linkage, and drive both intercellular and extracellular signaling [10, 11]. The integrin α subunit is crucial for selective molecule recruitment and plays a vital role in activating the Ras/MEK/ERK pathway [12, 13]. Recently, it was reported that ITGA1 was present in many kinds of tumors. For instance, inhibiting the expression of ITGA1 gene resulted in reduction of cell adhesion and invasion of breast cancer in mice [14]. Mice deleting ITGA1

gene showed longer survival with smaller tumors, and deletion of ITGA1 gene reduced cell proliferation, adhesions and increased cell death in lung cancers [15, 16].

Previous investigations have confirmed blocking Grb2 signaling or ITGA1 gene could inhibit cell proliferation, adhesion and invasion [17]. In this study, we explored the expression level of Grb2 in different cancer cells and the role of Grb2 in cancer growth, as well as its underlying mechanism. Our result showed that silencing Grb2 by siRNA reduced cancer proliferation through reducing the expression of ITGA1 and inhibiting the phosphorylation of ERK. Interestingly, simultaneous inhibition of Grb2 and ITGA1 resulted in a greater inhibition of phosphorylated ERK than either inhibition of Grb2 or ITGA1, and thus triggered apoptosis in Hela cells and Jurkat cells. The current findings show that Grb2 plays a vital role in regulating tumor proliferation and targeting Grb2 and ITGA1 pathway could be a promising strategy for cancer therapy.

Materials and methods

Data acquisition

Microarray gene expression data for 12 cell lines were obtained from NCBI Gene Expression Omnibus (GEO) (www.ncbi.nlm.nih.gov/geo/). The protein expression data for 11 cell lines were obtained from The ProteomeXchange Consortium (www.proteome xchange.org), Proteomecommons (http://proteomecommons.org/) and Proteomics DB (https://www.proteomicsdb.org/). Z score of per sample for Grb2 were calculated by median value of total samples.

Cell culture

MCF7, Hela, A549 and Jurkat cells were obtained from American Type Culture Collection (ATCC). All cells were grown in RPMI medium (GIBCO) with 10% FBS (GIBCO) and 1% penicillin/streptavidin (GIBCO), and maintained at 37°C in a humidified atmosphere at 5% CO_2.

siRNA transfection

For siRNA transfection, 2×10^5 cells per well were plated in a 6-well plate. After cells attached for 24 hours, Grb2, ITGA1 and control siRNA (RiboBio, Guangzhou RiboBio Co., Ltd.) were added in cells seeded in the 6-well plates for 6 hours at 37°C in a CO_2 incubator. After transfection, cells were supplemented with RPMI containing FBS and cultured at 37°C/5% CO_2 for up to 48 hours before harvest and protein extraction.

Western blot analysis

The Jurkat cells and HeLa cells with specific treatment were harvested and the protein supernatants were isolated using cell lysis buffer (Cell Signaling Technology; #9803) with added phenylmethylsulfonyl fluoride (PMSF). The extracted proteins were qualified by the BCA method. The total protein content (30 mg) from cell lysates were resolved by 10% SDS-PAGE, and transferred to a 0.45-mm nitrocellulose membrane (Millipore) for 1 hour. The membranes were washed with TBS-T containing 5% (w/v) BSA. The membranes were incubated overnight with specific Grb2 (Cell Signaling Technology), GAPDH (Cell Signaling Technology), ITGA1 (Abcam), ERK (Cell Signaling Technology), phosphor-ERK (Cell Signaling Technology), cleaved caspase-3 (Cell Signaling Technology) and cleaved PARP (Cell Signaling Technology) were exposed to secondary antibodies coupled to horseradish peroxidase for 2 hours at room temperature. The membranes were then washed three times with TBS-T at room temperature. Chemiluminescent signals were generated by the SuperSignal West Pico Trial Kit (Thermo Fisher Scientific Inc.) and detected using the Vilber Lourmat imaging system (Vilber Lourmat Corporation, Torcy, France).

Cell viability assay

Cell proliferation was determined using the CellTiter-Blue Cell viability Assay (Promega). Cells were seeded in a 96-well plate with density of the optimized cell number (2,000 cells/well). After 24 hours of seeding, cells were treated with siRNA at indicated working concentration. Cells were incubated for another 48 hours and then cell viability was measured using a fluorescence microplate reader (Sunrise Remote, Tecan Austria GmbH, Grödig, Austria).

LC-MS/MS

LC-MS/MS experiment was carried out on Thermo Q Exactive (Thermo Fisher Scientific,

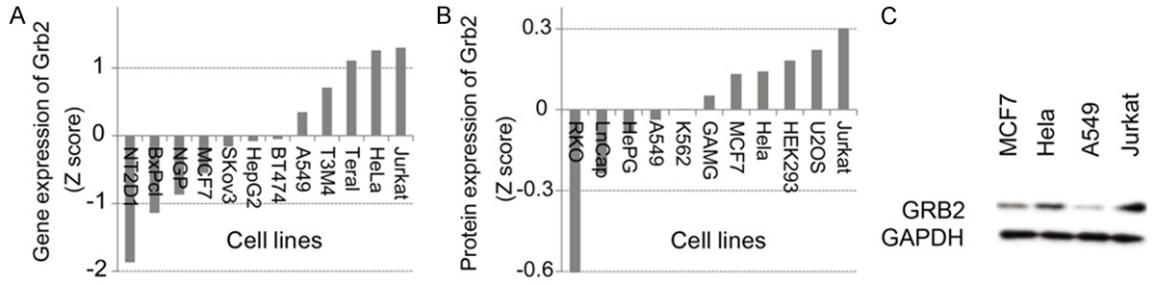


Figure 1. Grb2 expression level in different cancer cell lines. A. Gene expression of Grb2 in 12 cell lines. B. Protein expression of Grb2 in 11 cell lines. C. Western blot analysis for expression of Grb2 in several cancer cell lines.

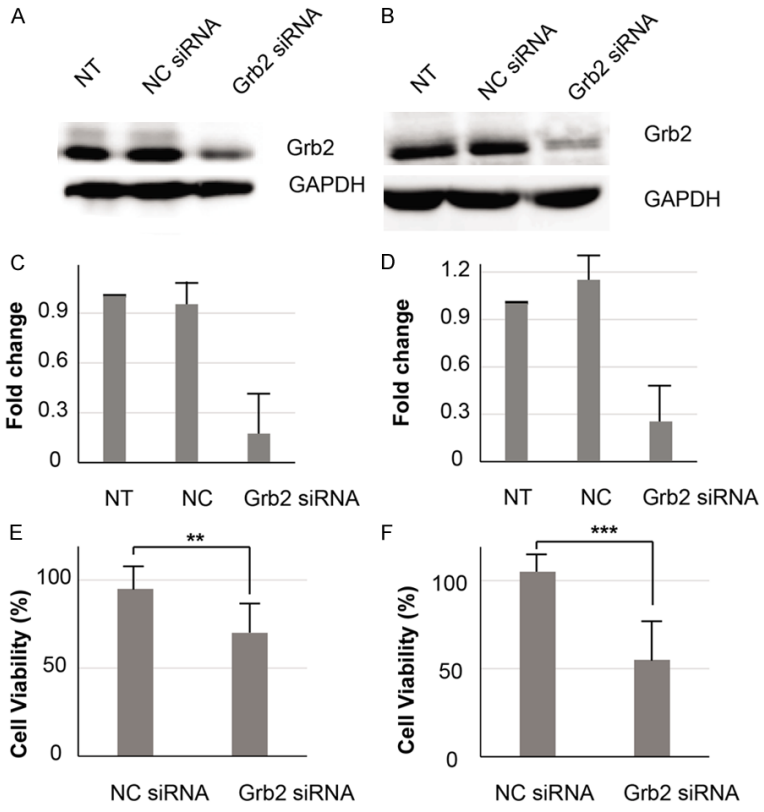


Figure 2. Knockdown of Grb2 suppresses cell viability of Hela cells and Jurkat cells. Protein expression of Grb2 after treatment of siRNA or negative control (NC) siRNA in Hela cells (A) and Jurkat cells (B). (C, D) Bar graphs of western blot analysis from panel (A) and (B), respectively. GAPDH was utilized as control. (E, F) Cell viability assessment after Grb2 siRNA treatment for 48 hours on Hela cells and Jurkat cells, respectively. Data are presented as mean ± S.E.M. (n=3). ***P*<0.01, ****P*<0.001.

San Jose CA). The raw MS spectra were processed with MaxQuant version 1.3.0.5. The MS/MS spectra were searched against the UniProt human database (released on 11 December, 2013 and containing 88,473 protein sequences), supplemented by frequently observed contaminants, and concatenated with reversed versions of all sequences.

Apoptosis detection

Flow cytometric analysis was performed to determine the percentages of apoptotic cells. One hour after cold 70% ethanol treatment, cells underwent 1-h digestion with 10 mg/ml RNase A at 37°C. Cells were then stained with propidium iodide (PI, 200 mg/ml), followed by flow cytometric analysis (Aria II, BD Biosciences). Cells (1×10^5) were counted for each sample. The percentages of apoptotic cells were presented as means ± S.E.M. in Figures.

Software support and statistical analysis

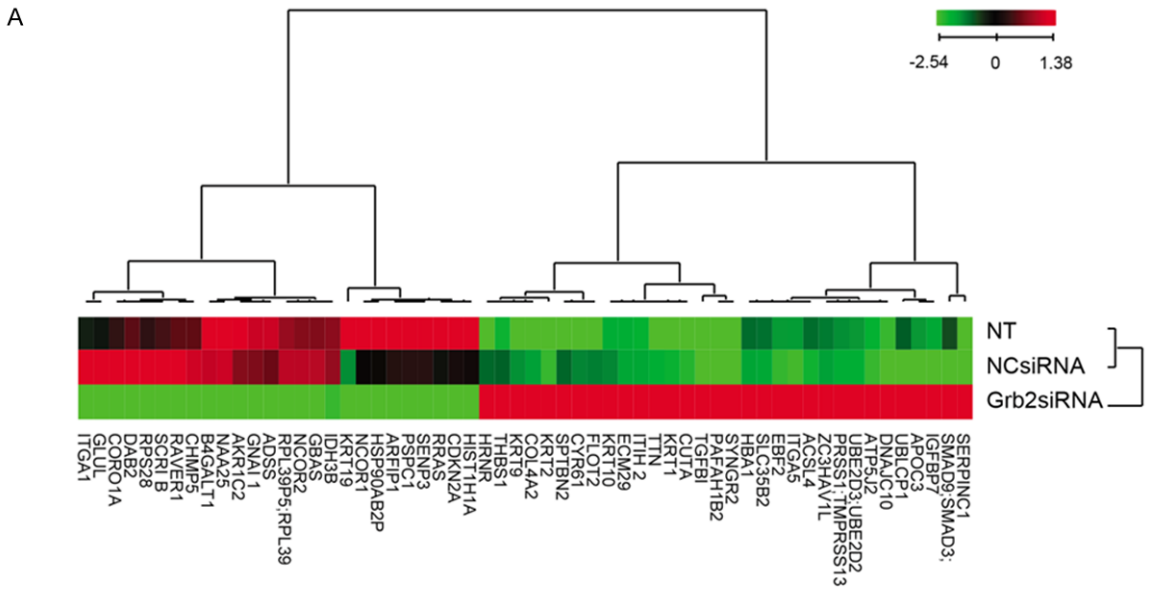
Hierarchical clustering of protein expression profile was carried out using QCanvas [18]. All images were formatted for optimal presentation using Adobe Illustrator CS4 (Adobe Systems). To determine statistical significance, *P*-value from t-statistic was calculated.

Result and conclusion

Expression level of Grb2 in different cancer cell lines

Growth factor receptor bound protein 2 (Grb2) is involved in tumorigenesis and tumor growth.

A



B

	Category	Term	Kappa
1	GOTERM_BP_DIRECT	positive regulation of cell migration	1.00
2	GOTERM_BP_DIRECT	negative regulation of apoptotic process	0.73
3	GOTERM_BP_DIRECT	positive regulation of epithelial to mesenchymal transition	0.60
4	KEGG_PATHWAY	Proteoglycans in cancer	0.60
5	KEGG_PATHWAY	Phagosome	0.60
6	GOTERM_MF_DIRECT	integrin binding	0.60
7	GOTERM_BP_DIRECT	positive regulation of protein phosphorylation	0.60
8	GOTERM_BP_DIRECT	response to hypoxia	0.60
9	GOTERM_BP_DIRECT	positive regulation of transcription, DNA-templated	0.55
10	KEGG_PATHWAY	TGF-beta signaling pathway	0.55
11	KEGG_PATHWAY	Endocytosis	0.55
12	GOTERM_BP_DIRECT	cell cycle arrest	0.55
13	GOTERM_CC_DIRECT	cell surface	0.55

Figure 3. Proteomic analysis of protein regulation under Grb2 siRNA treatment in Hela cells. A. Clustering of differential expressed proteins. Red, upregulation; Green, downregulation. B. GO enrichment analysis and KEGG pathway analysis for differential expressed proteins by using the DAVID database ((http://david.ncifcrf.gov/).

In this study, the gene expression and protein expression levels of Grb2 were analyzed in diverse cancer cell lines (**Figure 1A**, **1B**). The Grb2 showed a diverse expression pattern across cell lines. Especially, Jurkat and Hela cells showed a highly Grb2 expression in both protein and gene level. To confirm the varied expression of Grb2 in cancer cells, we used western blot to measure the expression level of Grb2 in MCF7, Hela, A549 and Jurkat cells (**Figure 1C**). As a result, highly expressed Grb2 was observed in Hela cells and Jurkat cells, which indicated that Grb2 may be involved in carcinogenesis in Grb2 highly expressed cells.

Proteomics study for Grb2

To systematically understand the function of Grb2 in cancer cells, we analyzed proteomic profiles of Grb2 siRNA treated Hela cells. A total of 62 differentially expressed proteins were identified (**Figure 3A**). To further investigate the function of the differential expressed proteins, we analyzed GO function and KEGG pathways through DAVID (https://david-d.ncifcrf.gov/). Those proteins were significantly involved in cell migration, apoptotic process, integrin binding, and cell cycle (**Figure 3B**). This suggested that upregulation of Grb2 levels may led to increased migration and cell proliferation in Grb2 highly expressed cancer cells.

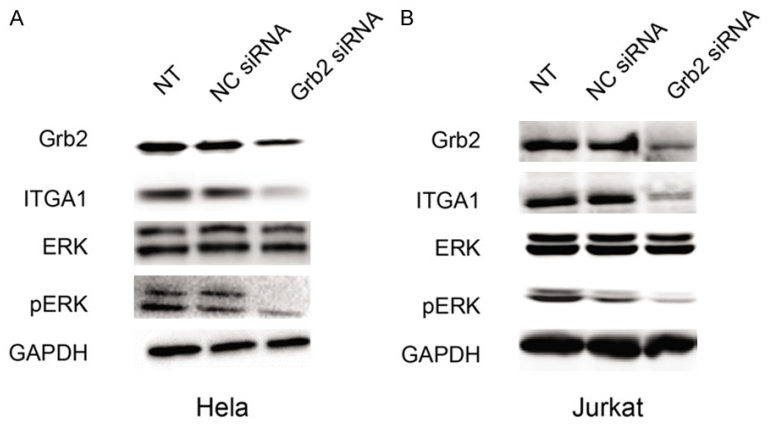


Figure 4. ITGA1 and ERK expression patterns after treatment with Grb2 siRNA or negative control siRNA in Hela cells and Jurkat cells. A. ITGA1 and ERK expression in Hela cells treating with Grb2 siRNA or NC siRNA. B. ITGA1 and ERK expression in Jurkat cells treating with Grb2 siRNA or NC siRNA. Western blot analysis was performed to detect the expression level of ITGA1, phosphorylated and total ERK protein.

Knockdown of Grb2 reduces cell proliferation

Previous study has found that Grb2 is a critical protein in cancer cell growth through MAPK signals and intracellular signals. To determine the generality of the impact of Grb2 in regulating cell growth, we adopted the cell viability assay. Grb2-specific small interfering RNAs (siRNAs) or its corresponding control siRNA were introduced into Hela cells and Jurkat cells and the efficiency of Grb2 siRNAs also tested (**Figure 2A-D**). As a result, knockdown of Grb2 in Hela cells and Jurkat cells exhibited significant decreased cell proliferation (**Figure 2E**, **2F**). These data strongly indicated that knockdown of Grb2 in Grb2 highly expressed cancer cells markedly decreased their proliferation.

Grb2 expression is positively correlate with ITGA1 and ERK activation

The preponderance of evidence continues to indicate that Grb2 has a critical role in mitogen activated protein kinase (MAPK) signaling. Grb2 activates Ras/ERK signaling and downstream transcription factors, and subsequently promotes the growth and inhibits the differentiation of tumor cells [1, 2]. The previous study also has found that the functions of integrin alpha 1 (ITGA1) play a pivotal role in cancer cells proliferation and malignant transformation [16]. Interestingly, ITGA1 (integrin binding pathway) was found to be significantly correlated with Grb2 knockdown response in cancer cells through our proteomic data analysis (**Figure 3A**, **3B**). To evaluate whether the expression of ITGA1 is affected by the change of Grb2 expression, we performed siRNA-mediated downregulation of Grb2 and evaluated the expression of ITGA1 by western blotting. As a result, the expression of ITGA1 was markedly decreased following Grb2 downregulation in Hela cells and Jurkat cells (**Figure 4A**, **4B**). In addition, downregulation of Grb2 led to a significant inhibition of phosphorylated ERK in Hela cells and Jurkat cells (**Figure 4A**, **4B**). Taken together, these results indicated that suppression of Grb2 reduced cancer proliferation through downregulating the expression of ITGA1 and inhibiting ERK signaling pathway in cancer cells highly expressing Grb2.

Synergistic effort of Grb2 and ITGA1 inhibition through ERK pathway

To further test whether ITGA is involved in the inhibition of ERK signaling to attenuate cell death, we downregulated ITGA1 using siRNA in Hela cells and Jurkat cells highly expressing Grb2. As expected, inhibition of ITGA1 could reduce the activation of ERK signaling in Hela cells and Jurkat cells (**Figure 5A**). In addition, simultaneous inhibition of Grb2 and ITGA1 resulted in the inhibition of ERK activation and the increase of cleaved caspase-3 and PARP, suggesting inhibition of both Grb2 and ITGA1 can promote apoptosis of cancer cells (**Figure 5A**).

To further confirm our hypothesis, we performed flow cytometric analysis to evaluate apoptosis in the presence or absence of ITGA1 siRNAs in Hela cells and Jurkat cells. As shown, in the presence of Grb2 siRNA, ITGA1 siRNA treatment significantly increased the percentage of sub-G_0/G_1 cell population compared with ITGA1 negative control siRNA (**Figure 5B**), suggesting inhibition of ITGA1 can potentiate the

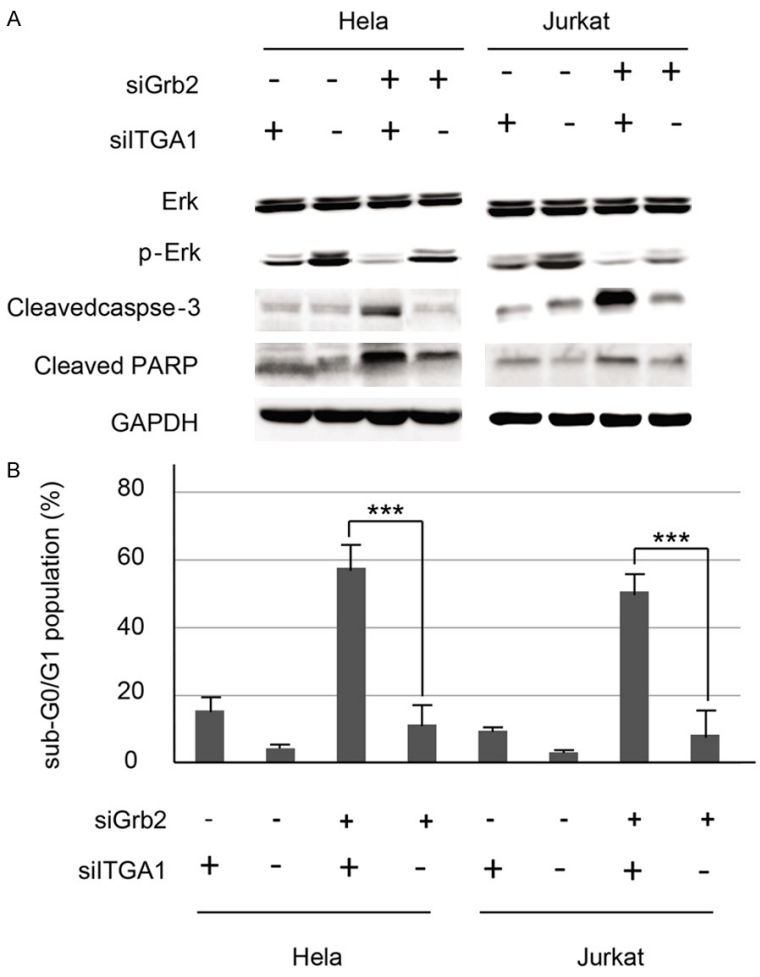


Figure 5. Synergistic effect of Grb2 and ITGA1 inhibition through ERK signaling. A. Hela cells and Jurkat cells were treated with Grb2 siRNA in the absence or presence of ITGA1 siRNA. Western blot analysis for expression of phosphorylated and total ERK, cleaved caspase-3 and cleaved PARP. B. The percentages of the sub-G0/G1 population were determined by flow cytometry in Hela cells and Jurkat cells. Data are represented as mean ± S.E.M. (n=3). ***P<0.001.

proapoptotic effect induced by Grb2 inhibition through ERK signaling pathway.

Discussion

It has been reported that the overexpression of receptor tyrosine kinases, overexpression of downstream proteins, including Grb2, may induce the upregulation of signaling pathways associated with cell transformation [19]. It also has demonstrated that upregulation of Grb2 is known to be associated with intracellular growth and proliferation related signaling cascades [8, 20, 21]. The previous studies proved that Grb2 plays a pivotal role in tyrosine kinase-mediated signal transduction including linking receptor tyrosine kinases to the Ras/mitogen-activated protein kinase (MAPK) pathway, which is implicated to result in oncogenic outcome. Overexpression of Grb2 protein in the fibroblast cell line NIH 3T3, potentiated the activation of mitogen-activated protein kinase (MAPK) [22]. In the present study, we verified that the expression level of Grb2 was varied in diverse cancer cells and it can promote cancer growth in cancers overexpressing Grb2. Knockdown of Grb2, the cell proliferation was distinctly decreased. Moreover, the activation of ERK is reduced following Grb2 silence. It indicated that Grb2 contributes to cancer cell proliferation and malignant transformation mediated by MAPK signaling pathway.

In our proteomic analysis, we found cell migration, apoptotic process, integrin binding, cell cycle were significantly correlated with Grb2 knockdown. In order to further clarify the underlying mechanism by which Grb2 promotes tumor growth by the ERK pathway and integrin pathway, we first observed the expression of ITGA1 and ERK activation. Decreased ERK phosphorylation and ITGA1 expression were found in Grb2 silenced cancer cells. In addition, treatment with the ITGA1 siRNA and Grb2 siRNA in Grb2-overexpressing cells resulted in the inactivation of ERK and increase of cell apoptosis. The above findings implied that the synergistic anticancer effect of Grb2 and ITGA1 on Grb2 upregulated cancer cells through ERK inhibition.

Taken together, we provided evidence that silencing Grb2 attenuates the activation of ERK pathway in cancer cells, ITGA1 and Grb2 has a synergistic affection to cancer mediated by ERK signaling. Our findings indicate that targeting Grb2 and ITGA1 pathway may be a promis-

ing strategy to strengthen the anti-tumor effect of current compounds for cancer therapy.

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Disclosure of conflict of interest

None.

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