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ORIGINAL ARTICLE

GRAIL inhibits the growth, migration and invasion of lung adenocarcinoma cells by modulating STAT3/C-MYC signaling pathways

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Summary

Purpose: *To explore the role and possible regulatory mechanisms of GRAIL in lung adenocarcinoma cells.*

Methods: *Online databases were used to analyze the expression and prognosis of GRAIL in patients with lung adenocarcinoma. Lung adenocarcinoma cells (A549 and H1299) were transfected with small interfering RNA or infected with lentivirus. The constructed cell models were validated by real-time fluorescence quantitative PCR. Cell proliferation ability was detected by CCK8 assay, cell migration and invasion ability were detected by scratch assay and Transwell assay. Suppressed hallmark gene sets of GRAIL in lung adenocarcinoma was performed by GeneSet Enrichment Analysis. The mRNA and protein level of C-MYC were detected by real-time fluorescence quantitative PCR and western blot. The protein level of STAT3 and phosphorylated STAT3 were detected by western blot.*

Results: *GRAIL is poorly expressed in lung adenocarcinoma tissue. Down-regulation of GRAIL is associated with poor prognosis of lung adenocarcinoma patients. GRAIL expression is negatively correlated with the proliferation, migration, and invasion of lung adenocarcinoma cells. GRAIL inhibits activation of STAT3 and transcription of C-MYC.*

Conclusions: *GRAIL inhibits the growth, migration and invasion of lung adenocarcinoma cells. GRAIL might play an anti-cancer role through regulating STAT3/ C-MYC signaling pathways.*

Key words: *C-MYC, GRAIL, invasion, migration, proliferation, lung adenocarcinoma*

Introduction

Globally, lung cancer shows the highest incidence and mortality rate among all types of tumors [1]. So far, lung cancer has been a serious threat to people's health. Lung cancer can be divided into small cell lung cancer and non-small cell lung cancer (NSCLC). As a type of NSCLC, the incidence of lung adenocarcinoma is also high. When diagnosed, many patients have already advanced-stage disease. Traditional treatment methods, targeted therapies and immunotherapies have improved the therapeutic effective rate for patients with lung adenocarcinoma [1,2]. However, there is still a certain number of patients who do not benefit from these treatments. Drug resistance in the treatment of lung adenocarcinoma also contributes to the difficulty of treatment [3]. Therefore, molecular-level research on the occurrence and development of lung adenocarcinoma may significantly improve its clinical treatment effects in the future [4].

GRAIL, or RNF128, is an E3 ubiquitin ligase [5]. Unlike other ubiquitin ligases, GRAIL was involved in the endocytosis process of transmembrane proteins [6]. GRAIL was highly expressed in lung, liver and other tissues. Although GRAIL has

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been reported to participate in the development of tumors [7,8], its role and mechanism in lung adenocarcinoma remain unclear. Thus, this study reveals the molecular mechanism of RNF128 in lung adenocarcinoma, and provides a promising new idea for the treatment of NSCLC.

Methods

Reagents and main instrument

Human lung adenocarcinoma A549 and H1299 cell lines were purchased from ATCC (USA, CCL-185 and CRL-5803). The 1640 culture medium was purchased from Kaiji Biology (China, KGM31800N-500). Fetal bovine serum (FBS) was purchased from Gibco (USA, 10099141). CCK8 kit was purchased from Dojindo (Japan, CK04). TransIT-X2 transfection reagent was purchased from Mirus (MIR6004, USA). Actin antibody, C-MYC antibody, phosphorylated STAT3 antibody and STAT3 antibody were purchased from CST (USA, 3700T, 5605, 9145, 4904). GRAIL antibody was purchased from Santa Cruz (SC-515110, USA). Rabbit antibody and rat antibody were purchased from CST (7074, 58802, USA). 7300 Fast Real-time fluorescence quantitative PCR instrument was purchased from American Applied Biosystems; The SpectraMax Multifunctional marker was purchased from Molecular Devices, USA; Transwell Chamber was purchased from Corning.

Expression analysis, survival analysis and GeneSet enrichment analysis

The expression of genes in the tissue was analyzed by the online website (http://gepia.cancer-pku.cn; https://www.ncbi.nlm.nih.gov/geo/geo2r). Survival analysis of genes was performed using the online site: https://kmplot.com.

GeneSet Enrichment Analysis (GSEA) was performed on lung adenocarcinoma tumors tissues and normal lung tissues in TCGA database. The data analysis was used for the classification version 2.10.1 package in R. Statistical calculations were performed using the Student's t-test.

Cell culture and transfection experiment

Lung adenocarcinoma cells were cultured in 1640 medium containing 10% FBS in an incubator at 37°C and 5% CO_2. 3×10^5 cells were spread into a 6-well plates, and each well was added with 2ml 10% FBS in 1640 medium. After 24 h, cells were transfected with TransIT-X2 and siRNA.

Establishment of stable cell line model

ShRNA expression vector of RNF128 was constructed by pLV3ltr-U6. pLV3l-CMV was used to construct the overexpression system. Lung adenocarcinoma cells were infected with lentivirus and screened with purinomycin 72 h later.

CCK8 experiment

The CCK8 stock solution was diluted in serum-free 1640 medium to one-tenth of the final treatment volume. The cells were plated in a 96-well plates, and the culture medium was replaced with the test liquid at each detection time. The absorbance of test liquid at 450 nm was measured after an hour incubation in a 37°C incubator.

Scratch experiment

The cells were cultured in 6-well plates to achieve 80-90% confluence. The tip of a 10ul sterile spear was used to draw a vertical scratch of the same thickness in the cells. The width of the scratch was observed at 0 h and 24 h after scratching. ImageJ was used to compare the change in the width of the scratch.

Transwell assay

In the Transwell chamber, cells resuspended in serum-free medium were placed in the upper chamber. In

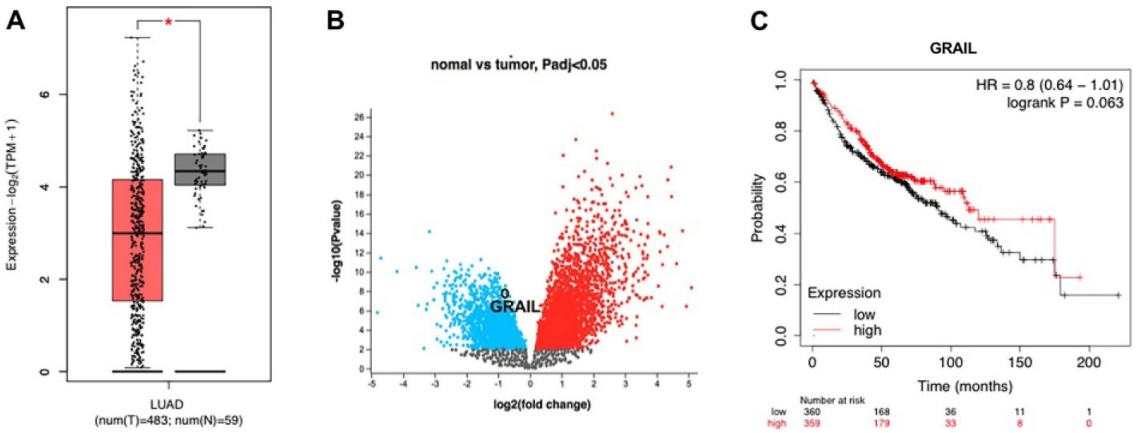

Figure 1. Expression and prognosis of GRAIL in lung adenocarcinoma. **A:** The expression of GRAIL in lung adenocarcinoma of the TCGA database, *p<0.05. **B:** The expression of GRAIL in the GSE116959 data set of GEO database, Padj<0.05. **C:** The relationship between the expression level of GRAIL and prognosis in patients with lung adenocarcinoma in the TCGA database, p=0.063.

the lower chamber, 1640 medium containing 20% FBS was placed. After incubation for 12 or 24 h, 4% paraformaldehyde was used for 30 min, then the Transwell chamber was washed three times with phosphate buffered saline (PBS). Crystal violet dye was used for 15 min. Photographs were taken under an optical microscope. In the invasion experiment matrix glue was added at the upper chamber.

Real-time fluorescence quantitative PCR and Western-blot assay

The extracted RNA was quantified and then reversed-transcribed, and the product was amplified by PCR. GAPDH was selected as internal reference gene and the relative quantification was calculated.

According to the protocol, antibodies were diluted to the antibody solutions. The primary antibody was incubated at 4°C overnight. The membrane was washed by PBS with tween-20 for 3 times. Then, the secondary antibody was added and incubated at room temperature for one hour. The obtained protein bands were analyzed by Image J.

Statistics

Imge J, GraphPad Prsim 8 and Image Lab were used for statistical analyses. The experiments were performed in triplicate and measured. The data were expressed as mean ± standard deviation. T-test or one-way ANOVA between groups were used for analysis, and $p<0.05$ was considered statistically significant. * means $p<0.05$, ** means $p<0.01$, and *** means $p<0.001$.

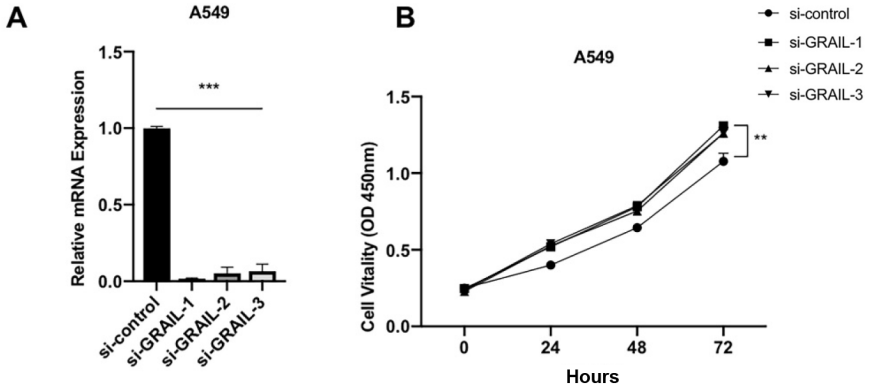

Figure 2. After siRNA targeting gene GRAIL, the proliferation rate of A549 cells was increased. **A:** Validation efficiency of siRNA targeting gene GRAIL, ***p<0.001. **B:** The proliferation rate in A549 cells, **p<0.01.

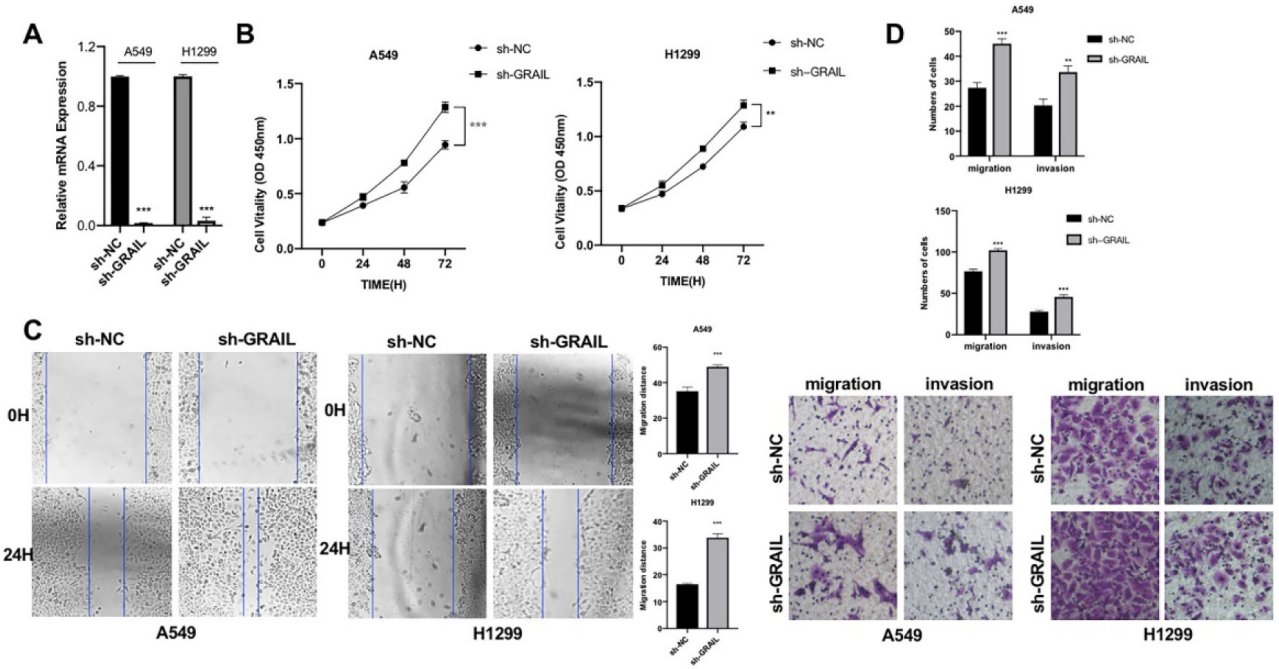

Figure 3. Lung adenocarcinoma cells that knocking down GRAIL have increased proliferation, migration and invasion ability. **A:** Validation efficiency of knockdown GRAIL cell lines, p<0.001. **B:** The proliferation rates in A549 and H1299 cells, p<0.01. **C:** The ability of wound-healing in A549 and H1299 cells, p<0.01. **D:** The ability of migration and invasion in A549 and H1299 cells, p<0.01.

Results

The expression of GRAIL in lung adenocarcinoma

GEPIA2 was used to analyze the expression levels of GRAIL in 483 lung adenocarcinoma and 59 normal lung tissues in the TCGA database. Compared to normal tissue, GRAIL was found to be low in lung adenocarcinoma tissue (Figure 1A, p<0.01). In the GSE116959 data set of GEO database (transcriptome analysis of 57 lung adenocarcinoma tissue samples and 11 adjacent normal lung tissue samples), we also found that the GRAIL expression in lung adenocarcinoma was less than its expression in adjacent normal lung tissue (Figure 1B, Padj<0.05).

Next, Kaplan-Meier Plotter online was used to analyze the relationship between the expression level of GRAIL and prognosis in patients with lung adenocarcinoma in the TCGA database. Based on the median of GRAIL expression level, lung adenocarcinoma patients were divided into high-GRAIL group or low-GRAIL group. It was found

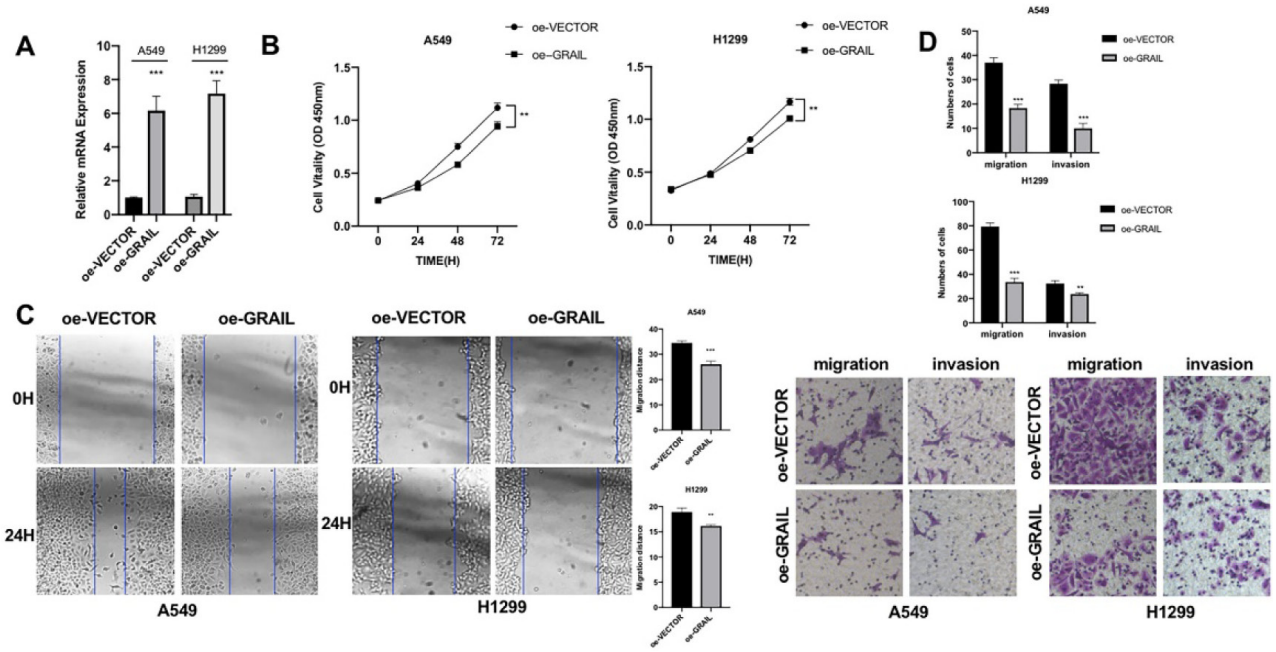

Figure 4. After overexpression of GRAIL, Lung adenocarcinoma cell proliferation, migration and invasion ability were reduced. **A:** Validation efficiency of GRAIL overexpression cell lines, ***p<0.001. **B:** The proliferation rates in A549 and H1299 cells, **p<0.01. **C:** The ability of wound-healing in A549 and H1299 cells, p<0.01. **D:** The ability of migration and invasion in A549 and H1299 cells, p<0.01.

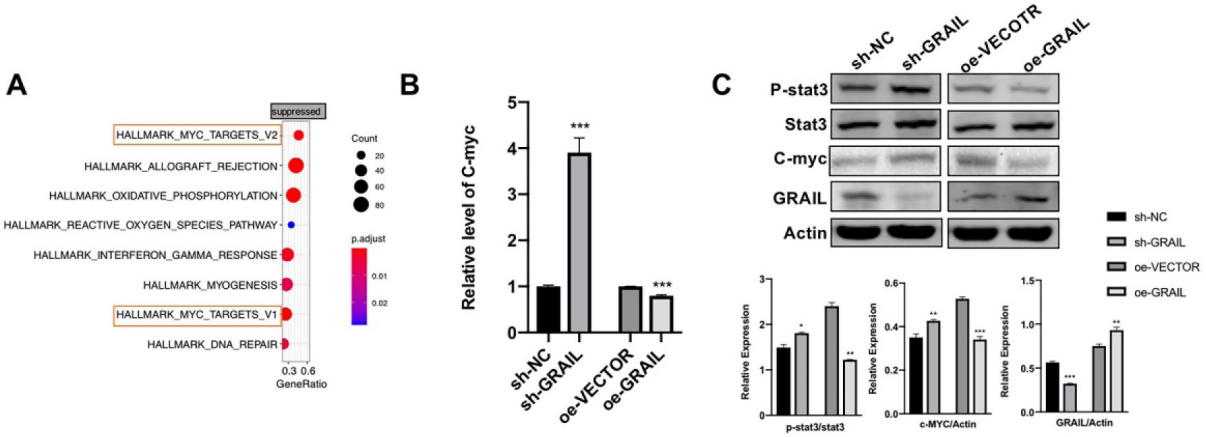

Figure 5. GRAIL inhibited activation of STAT3 and transcription of C-MYC. **A:** Suppressed hallmark gene sets of GRAIL in lung adenocarcinoma. Count is the number of core genes, GeneRatio is the ratio between Count and setSize. **B:** The mRNA level of C-MYC in A549, ***p<0.001. **C:** The protein levels of STAT3, phosphorylated STAT3 and C-MYC in A549, **p<0.05.

that patients in low-GRAIL group were associated with lower survival rates (Figure 1C, p<0.01). The median survival time of patients in the GRAIL low-expression group and high-expression group was 90 months and 112.67 months, respectively.

Knocking down the GRAIL expression, the proliferation rate of A549 cells increased

After using three kinds of small interfering RNA (siRNA) to interfere with the expression of GRAIL, the expression levels of GRAIL in lung adenocarcinoma A549 cells decreased significantly (Figure 2A, p<0.001). Via CCK8 experiment, we measured the proliferation ability of cells. The interference of GRAIL expression significantly enhanced the viability of A549 cells (Figure 2B, p<0.01).

We further constructed stable knockdown GRAIL cell lines by lentiviruses in lung adenocarcinoma A549 and H1299 cells (Figure 3A, p<0.001). Compared with the control group, the proliferation rates of A549 and H1299 cells increased (Figure 3B, p<0.01). At the same time, we analyzed the migration and invasion potential of lung adenocarcinoma cells through the scratch method and transwell method. Compared with the control group, the potential of migration and invasion was increased (Figure 3C, 3D, p<0.01). This demonstrated that the reduced GRAIL expression led to increased proliferation rate of lung adenocarcinoma cells.

Increased GRAIL expression leads to decreased cell proliferation, migration and invasion potential

A549 and H1299 cells were infected with lentivirus to increase GRAIL expression levels. The overexpression efficiency was detected by Real-time fluorescence quantitative PCR (Figure 4A, p<0.001). Compared with the control group, the cell proliferation rate of the experimental group was reduced (Figure 4B, p<0.01), and the invasion and migration ability was weakened (Figure 4C, 4D, p<0.01). The results proved that GRAIL inhibited lung adenocarcinoma cells' proliferation, migration and invasion ability.

GRAIL affects STAT3/ C-MYC pathway

GeneSet Enrichment Analysis (GSEA) was performed on lung adenocarcinoma tumor tissues and normal lung tissues in TCGA database. The suppressed hallmark gene sets of GRAIL in lung adenocarcinoma was shown by Bubble chart. The result revealed that the MYC target gene, the downstream gene of C-MYC, was suppressed. We then investigated whether the MYC pathway is inhibited (Figure 5A).

In A549 cells, compared with the control group, the mRNA level of C-MYC was increased after the expression of GRAIL was decreased. In turn, with increasing GRAIL expression level, C-MYC was decreased (Figure 5B, p<0.001). Western blot experiment was used to detect the reported upstream gene of the C-MYC pathway. Changes in GRAIL expression did not convert the total amount of STAT3 protein. Compared with the control group, the phosphorylated STAT3 and C-MYC protein levels were increased when the expression of GRAIL was decreased, and decreased when the expression of GRAIL was increased (Figure 5C, p<0.05). In decreased stimulation of STAT3 signaling pathways, C-MYC protein levels were down-regulated. GRAIL may affect lung adenocarcinoma cell via STAT3/ C-MYC pathway.

Discussion

GRAIL has previously been reported to regulate lymphocyte function and produce cytokines. In this study, we first discovered the correlation between GRAIL and lung adenocarcinoma. Firstly, we analyzed the expression of GRAIL in lung adenocarcinoma tissues through the TCGA database and GEO database. The results showed that the expression of GRAIL was low in lung adenocarcinoma tissues compared to normal lung tissues. We also performed a statistical analysis on the survival rate of lung adenocarcinoma patients. Low-expression GRAIL in lung adenocarcinoma patients predicted low survival rate and poor prognosis.

By using small interfering RNA or lentivirus to transfect lung adenocarcinoma A549 and H1299 cells, we found that changes in GRAIL expression levels were associated with lung adenocarcinoma cell activity, migration potential and invasion ability. GRAIL affects the proliferation, migration and invasion of lung adenocarcinoma cells.

C-MYC is an important proto-oncogene, which plays a vital role in the occurrence and development of lung cancer [9]. STAT3 is involved in cytokine signal transduction and it is an important factor in the regulation of cell proliferation and apoptosis [10,11] and may promote the proliferation of tumor cells by up-regulating C-MYC. GRAIL may reduce the phosphorylation level of STAT3 to inhibit STAT3 activation. The reduction in STAT3 signal intensity will affect its downstream factors such as C-MYC. Thus, the transcription of C-MYC was down-regulated and the cell viability was affected. Our study found that GRAIL expression levels were negatively correlated with phosphorylated STAT3 and C-MYC protein levels, suggesting that GRAIL may exert a tumor sup-

pressor effect by inhibiting the STAT3/ C-MYC pathway.

Our experiments show that GRAIL plays an important role in lung adenocarcinoma via inhibiting the activation of STAT3 and the transcription of C-MYC. However, the above conclusions are limited to *in vitro* experiments. The role of GRAIL in lung adenocarcinoma needs further verification *in vivo*. In addition, the current research on the mechanism of GRAIL in lung adenocarcinoma is relatively shallow. How GRAIL exerts its anti-tumor effect in lung adenocarcinoma requires further exploration.

Acknowledgements

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Conflict of interests

The authors declare no conflict of interests.

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