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Original Article

GRIK3 deficiency promotes non-small cell lung cancer progression by the regulation of the UBE2C/CDK1/Wnt signaling pathway

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Abstract: Glutamate ionotropic receptor kainate type subunit 3 (GRIK3) is a predominant excitatory neurotransmitter receptor in the mammalian brain. While it is known that GRIK3 is involved in normal neurophysiologic processes, its biological functions in tumor progression are still poorly understood due to limited investigation. In this study, we reported for the first time that GRIK3 expression was downregulated in non-small cell lung cancer (NSCLC) tissues as compared to paracarcinoma tissues. Additionally, we observed that GRIK3 expression was strongly correlated with the prognosis of NSCLC patients. We also noted that GRIK3 suppressed the cell proliferation and migration capability of NSCLC cells, thereby inhibiting xenografts growth and metastasis. Mechanistically, GRIK3 deficiency increased the expression of ubiquitin-conjugating enzyme E2 C (UBE2C) and cyclin-dependent kinase 1 (CDK1), which resulted in the activation of the Wnt signaling pathway and enhanced NSCLC progression. Our findings suggest that GRIK3 plays a role in regulating NSCLC progression and that its expression may serve as an independent prognostic indicator for NSCLC patients.

Keywords: GRIK3, non-small cell lung cancer, Wnt signaling, UBE2C, CDK1

Introduction

Lung cancer is the leading cause of cancer-related deaths, responsible for 25% of all cancer deaths. Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all diagnosed cases of lung cancer worldwide [1]. Although targeted molecular therapies and immunotherapies have advanced in recent years, the 5-year survival rate for NSCLC remains at approximately 21%, highlighting its ongoing status as a significant medical challenge [2]. Given its high prevalence and mortality rate in both sexes, the development of novel and effective treatments for NSCLC is an urgent priority. Targeted molecular therapies, which are specific to tumor cells and have minimal adverse effects, have gained considerable interest in comparison to conventional treatments [3]. To improve the clinical outcomes of NSCLC patients, it is necessary to comprehend the molecular mechanisms underlying tumor progression and drug resistance and develop novel therapeutic targets.

GRIK3 is a member of the ionotropic glutamate receptor (iGluR) family, which acts as the first messenger and plays crucial roles in the neuroactive ligand-receptor interaction pathway [4]. Furthermore, GRIK3 is essential for synaptic potentiation, a critical process for memory and learning [5]. Previous evidence showed that GRIK3 participated in cancer development and was abnormally expressed in various types of cancer, including neuroblastoma, glioma, thyroid tumor, lung cancer, breast cancer, myeloma, and colorectal cancer [6-8]. Moreover, aberrant methylation of the *GRIK3* gene has been found in all the stages of lung adenocarcinoma, suggesting that *GRIK3* might be a novel epigenetic biomarker for diagnosis [9-11]. Studies have also shown that GRIK3 expression is associated with lymph node metastasis and can serve as an independent prognostic factor

for patients with gastric cancer [12]. However, the molecular mechanism underlying the impact of GRIK3 expression on malignant progression remains unclear.

Previous studies have shown that GRIK3 is involved in multiple signaling pathways related to breast cancer development, including epithelial-mesenchymal transition and cell proliferation [13]. In this study, we aimed to gain new insights into the role of GRIK3 in NSCLC progression. Our findings indicate a close correlation between GRIK3 expression and clinicopathological stages as well as patient prognosis in NSCLC. Additionally, we investigated the specific molecular mechanisms underlying tumor growth and metastasis associated with GRIK3 in NSCLC, thereby identifying novel therapeutic targets for the clinical treatment of this disease.

Materials and methods

Cell lines and reagents

Human NSCLC cell lines, namely A549 and NCI-H1299, were obtained from the Global Bioresource Center (ATCC). The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in Roswell Park Memorial Institute (RPMI)-1640 culture medium (Gibco, USA), which contained 10% fetal bovine serum (FBS, Gibco, USA). To establish A549 and NCI-H1299 cell lines with GRIK3, CDK1, and UBE2C overexpression, iCarTab Co. (China) was employed, and their validation was performed using Western blotting. Next, A549, and NCI-H1299 cells that were silenced for UBE2C were constructed by siRNA targeting (Ruibo Co., China). Wnt/β-catenin signaling inhibitor FH535 was obtained from MedChemExpress (USA).

Clinical specimens and information

We procured 30 human NSCLC tumor tissues from patients who underwent surgical treatment at the Guangzhou First People's Hospital. The patients were divided into two groups: recurrent (n=15) and non-recurrent (n=15), based on the outcomes of our follow-up visits. Additionally, we obtained the data of 518 patients from the TCGA databases (https://www.cbioportal.org/ and http://ualcan.path.uab.edu/index.html). All participants willingly consented to take part in the study, and we acquired their informed consent in written form, conforming to the guidelines of the Declaration of Helsinki. The Ethics Committee of Guangzhou First People's Hospital approved the study's ethical review process. All experiments were conducted in accordance with the aforementioned guidelines.

Cell proliferation and colony formation

Cell proliferation was determined using Cell Counting Kit-8 (CCK-8, Solarbio, China). Briefly, 2000 of A549 or NCI-H1299 cells were seeded in a 96-well plate and incubated with 10 μl of CCK-8 reagent after 0, 24, 48, and 72 hours, respectively. The rate of cell proliferation was measured using a microplate reader (Thermo Fisher, USA) to detect the absorbance at 450 nm. For the colony formation assay, 500 of A549 or NCI-H1299 cells were seeded in a 6-well plate and incubated with FBS free culture medium. After 10 days, the colonies were fixed with paraformaldehyde and stained with crystal violet. The colony numbers were counted and colony formation rates were calculated.

Quantified polymerase chain reaction (qPCR)

The quantification of mRNA levels was conducted by real-time PCR using an SYBR green dye (Thermo Fisher, USA), and GAPDH was used for the normalization. The primer sequences were listed as follows: Gli1 forward primer, 5'-AGCGTGAGCCTGAATCTGTG-3' and reverse primer, 5'-CAGCATGTACTGGGCTTTGAA-3'; Gli2 forward primer, 5'-CAGCATGTACTGGGCTTTGAA-3' and reverse primer, 5'-GCATGGAATGGTGGCAAGAG-3'; Wnt-3a, 5'-AGCTACCCGATCTGGTGGTC-3' and reverse primer, 5'-AGCTACCCGATCTGGTGGTC-3'; GAPDH forward primer, 5'-TGTGGGCATCAATGGATTTGG-3' and reverse primer 5'-ACACCATGTATTCCGGGTCAAT-3'.

Western blotting

The pre-treated A549 and NCI-H1299 cells were collected and lysed in a RIPA lysing buffer (Biyuntian, China) that contained protease and phosphatase inhibitor cocktails (Biyuntian, China). The collected proteins were then quantified and separated by electrophoresis on 10% SDS-polyacrylamide gels; thereafter, they were electrophoretically transferred to a methanol-pretreated PVDF membrane (Thermo Fisher, USA). The samples were blocked with 5% block-

ing buffer, and incubated with the following primary antibodies overnight at 4°C: anti-GRIK3 (ab233734, Abcam, UK), anti-β-catenin (ab-32572, Abcam, UK), anti-Wnt-3a (ab219232, Abcam, UK), anti-CDK1 (ab133327, Abcam, UK) and anti-UBE2C (ab252940, Abcam, UK). The blots were finally visualized with horseradish peroxidase (HRP)-conjugated secondary antibody.

Immunohistochemistry and immunofluorescence

Paraffin sections of NSCLC tissues were dewaxed and treated with sodium citrate antigen retrieval buffer. Next, the sections were blocked using a blocking buffer (Thermo, USA), and incubated with the following primary antibodies overnight at 4°C: anti-GRIK3 (ab233734, Abcam, UK), anti-Wnt-3a (ab219232, Abcam, UK), anti-CDK1 (ab133327, Abcam, UK) and anti-UBE2C (ab252940, Abcam, UK). This was followed by incubation with HRP-conjugated secondary antibodies. The nucleus was visualized using 4',6-diamidino-2-phenylindole (DAPI) or hematoxylin. Furthermore, the protein expression was quantified by Image J 6.0 or Image Pro Plus 6.0 software (USA).

Animal protocols

Pathogen-free 6-week-old female NOD-SCID mice were obtained from SPF Biotechnology Co. Ltd. All the animal experimental protocols were approved by the Ethics committee of the Guangzhou First People's Hospital. For the tumor growth assay, 1×10^6 vector or GRIK3 overexpression A549 cells were subcutaneously injected into the mice. The tumor volumes of the mice were recorded every day (n=6 in each group). The formula used to calculate the tumor volume was as follows: tumor volume = length × $width^2/2$. For tumorigenic and metastasis potential assays, 1×10^5 vector cells and A549 cells with GRIK3 overexpression were injected into the mice by a tail vein injection (n=9 in each group). After 20 days, the mice were sacrificed, and lung tissues were collected for hematoxylin-eosin (H&E) staining. Furthermore, the metastatic tumor nodules in lung tissues were counted. All the animal studies were conducted in accordance with the Public Health Service Policy and complied with the WHO guidelines for the humane use and care of animals.

Statistical analysis

All the data were presented as the mean ± SEM, and analyzed by GraphPad 7.0. Further, the statistical significance between the groups was calculated by Student's t-test for two groups and one-way ANOVA for three or more groups. The survival rates were evaluated using the Kaplan-Meier survival analysis. All the experiments in our study were carried out thrice independently. $^*P < 0.05$; $^{**}P < 0.01$; n.s no significant difference.

Results

Down-regulation of GRIK3 in NSCLC

To investigate the potential role of GRIK3 in the progression of NSCLC, we first evaluated its expression at the transcriptome level in 515 NSCLC tissues compared to 59 para-carcinoma tissues using the TCGA database. Intriguingly, we found a significant decrease in GRIK3 expression levels in NSCLC tumors as compared to para-carcinoma tissues (**Figure 1A**). Meanwhile, GRIK3 progressively downregulated with the stages of the clinical tumor, especially in stage IV tumor tissues (**Figure 1B**). Given the observed decrease in GRIK3 expression in NSCLC tumor tissues, we examined whether GRIK3 influenced the overall survival of NSCLC patients. We categorized 508 patients into high and low GRIK3 expression groups and found that patients with high GRIK3 expression had significantly longer overall survival (**Figure 1C**). To further investigate the potential tumor suppressive role of GRIK3, we collected 30 tumor tissues from clinical NSCLC patients categorized into recurrent (n=15) and non-recurrent (n=15) groups based on their follow-up visit. Immunohistochemistry was used to determine GRIK3 expression in these tissues. Consistent with our previous findings, a significant decrease in GRIK3 expression was observed in tumor tissues from the recurrent group compared to the non-recurrent group (**Figure 1D**). These results further support the hypothesis that GRIK3 may serve as a tumor suppressor in NSCLC. To confirm the tumor suppressor effect of GRIK3, we overexpressed GRIK3 in A549 and NCI-H1299 NSCLC cell lines (Figure S1A). Cell proliferation and tumorigenic potential were assessed using CCK-8/colony formation assay. We found that overex-

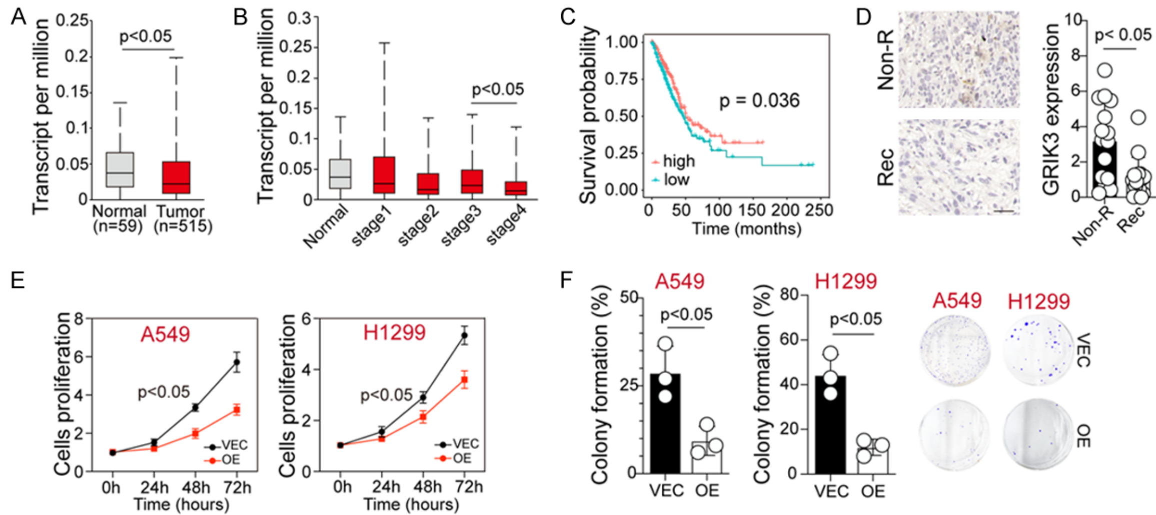

Figure 1. GRIK3 was down-regulated in NSCLC. A. The expression of GRIK3 in 515 NSCLC tissues and 59 para-carcinoma tissues from the TCGA database. B. The expression of GRIK3 in 59 para-carcinoma tissues and 515 NSCLC tissues (277 stage I, 125 stage II, 85 stage III and 28 stage IV). C. The overall survival of NSCLC patients who were divided into high GRIK3 (n=252) and low GRIK3 (n=253) groups was analyzed using the TCGA database. D. The immunohistochemistry of GRIK3 in NSCLC tissues from recurrent (n=15) and non-recurrent (n=15) patients. The scale bar was 50 μm. The expression intensity of GRIK3 was calculated. E. The relative cell proliferation of the vector cells and A549/NCI-H1299 cells with GRIK3 overexpression. F. The colony formation capability of vector cells and A549/NCI-H1299 cells with GRIK3 overexpression.

pression of GRIK3 significantly suppressed cell proliferation in A549 and NCI-H1299 cell lines (**Figure 1E**). Additionally, A549 and NCI-H1299 cells with GRIK3 overexpression showed decreased colony formation capability compared to the vector group (**Figure 1F**). Collectively, these findings provide evidence that GRIK3 exerts a tumor suppressive role in reducing the malignant progression of NSCLC.

GRIK3 suppressed Wnt/β-catenin signaling to inhibit NSCLC progression

Taking into consideration previous results regarding the negative correlation of GRIK3 expression with NSCLC progression, we aimed to elucidate the underlying mechanism of GRIK3-induced suppression of NSCLC progression. In this regard, 518 NSCLC patients were categorized into the low GRIK3 group (relative GRIK3 expression, 0~10, n=393) and the high GRIK3 group (relative GRIK3 expression, 10~1500, n=125). The mRNA expression profiles of 518 patients were analyzed by using the TCGA database. Differential gene expression was compared between the two groups, and a Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed to determine the main signaling pathways involved with GRIK3 (**Figure 2A**). We found that GRIK3 was strongly involved in modulating the Wnt and hedgehog signaling pathways, which are major pro-survival signals in tumor progression. To clarify the role of these signals in GRIK3-associated tumor progression, we measured the expression of Gli1, Gli2 (two hedgehog-regulated transcription factors), and Wnt-3a (an activator of the canonical Wnt signaling pathway) using quantified PCR in A549/NCI-H1299 cells with GRIK3 overexpression. Interestingly, we observed a decrease in the expression of Wnt-3a in A549/NCI-H1299 cells with GRIK3 overexpression compared to the vector cells (**Figure 2B**). However, there was no significant difference in Gli1 and Gli2 expression levels between the groups (**Figure 2C**). Western blot analysis consistently showed that Wnt-3a and β-catenin were downregulated at the protein level in GRIK3 overexpression cells compared to vector A549/NCI-H1299 cells (**Figure 2D**), suggesting a role for Wnt signaling in GRIK3-associated tumor progression. To confirm that GRIK3 downregulated Wnt/β-catenin signaling to suppress NSCLC progression, we treated A549/NCI-H1299 cells (vector and GRIK3 overexpression) with FH535, a Wnt/β-catenin signaling inhibitor, and assessed cell proliferation and colony formation. Blockade of Wnt/β-

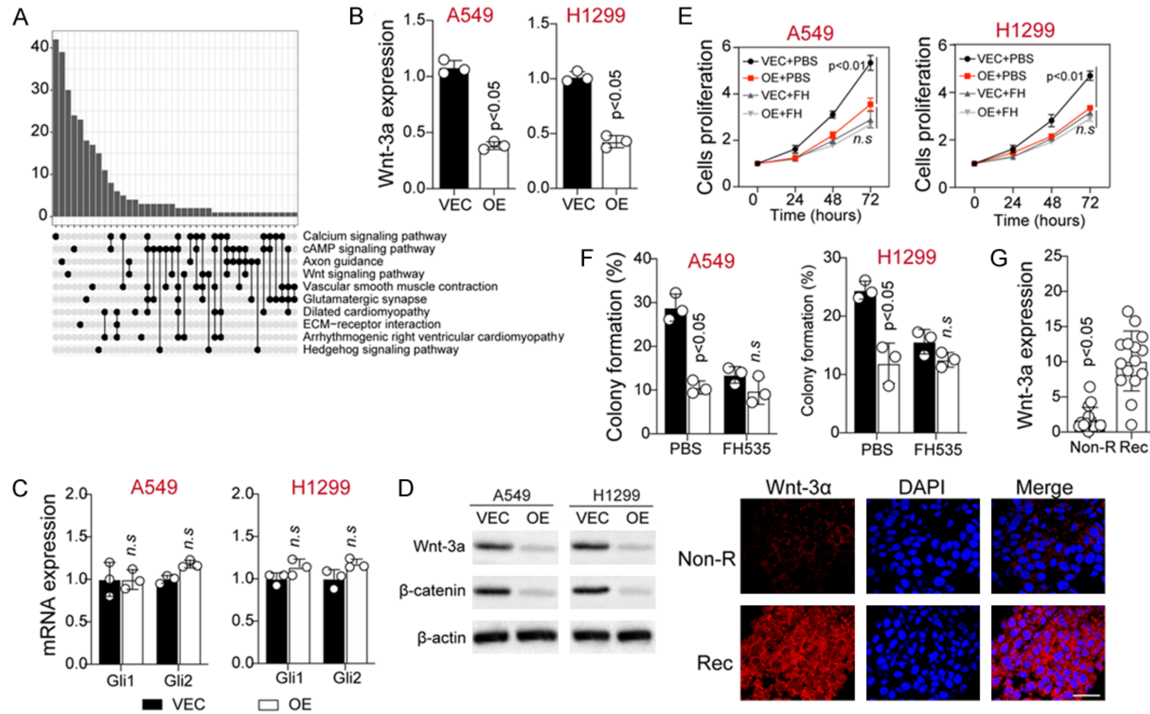

Figure 2. GRIK3 suppressed Wnt/β-catenin signaling to inhibit the progression of NSCLC. A. KEGG pathways analysis was performed in low GRIK3 expression (relative GRIK3 expression, 0~10, n=393) and high GRIK3 expression (relative GRIK3 expression, 10~1500, n=125) patients with NSCLC. B and C. Relative Gil1, Gli2 and Wnt-3a expression in vector and A549/NCI-H1299 cells with GRIK3 overexpression, as determined by quantified PCR. D. Western blotting of Wnt-3a and β-catenin in vector cells and A549/NCI-H1299 cells with GRIK3 overexpression. E. The relative cell proliferation of vector cells/A549/NCI-H1299 cells with GRIK3 overexpression that were treated with PBS or FH535 (10 nM). F. The colony formation capability of vector cells/A549/NCI-H1299 cells with GRIK3 overexpression that were treated with PBS or FH535 (10 nM). G. Immunofluorescence of Wnt-3a in NSCLC tissues in recurrent (n=15) and non-recurrent (n=15) patients. The scale bar was 50 μm. The expression intensity of GRIK3 was calculated.

catenin signaling abolished the tumor suppressive effects induced by GRIK3 (**Figure 2E** for cell proliferation and **Figure 2F** for colony formation), indicating that GRIK3 regulated NSCLC development in a Wnt/β-catenin signaling-dependent manner. We also examined the expression of Wnt-3a in clinical specimens and found a remarkable increase in Wnt-3a expression in tumor tissues from the recurrent group compared to the non-recurrent group (**Figure 2G**). Collectively, our results demonstrate that GRIK3 inhibits Wnt signals to regulate NSCLC progression.

GRIK3 deficiency contributed to UBE2C/CDK1/Wnt signaling activation

To investigate how GRIK3 controls the activation of the β-catenin/Wnt signaling pathway and suppresses NSCLC development, we compared the differentially expressed genes between high and low GRIK3 groups. We identified the top 30 downregulated genes in the high GRIK3 group and found that UBE2C was significantly decreased in NSCLC tissues of the high GRIK3 group (**Figure 3A**). Previous studies have shown that a deficiency of UBE2C reduces ovarian cancer malignancy and reverses cisplatin resistance by downregulating CDK1, which can mediate β-catenin activity by PDK1 [14]. Therefore, we hypothesized that GRIK3 downregulated Wnt/β-catenin signaling by suppressing the UBE2C/CDK1 axis. We performed Western blotting to examine the influence of GRIK3 on UBE2C and CDK1 expression in A549/NCI-H1299 cells, and observed that the overexpression of GRIK3 suppressed UBE2C and CDK1 expression in NSCLC cells (**Figure 3B**). To assess whether the UBE2C/CDK1 axis played a role in the regulation of Wnt/β-catenin signaling, we treated A549/NCI-H1299 cells with UBE2C and CDK1 siRNA for

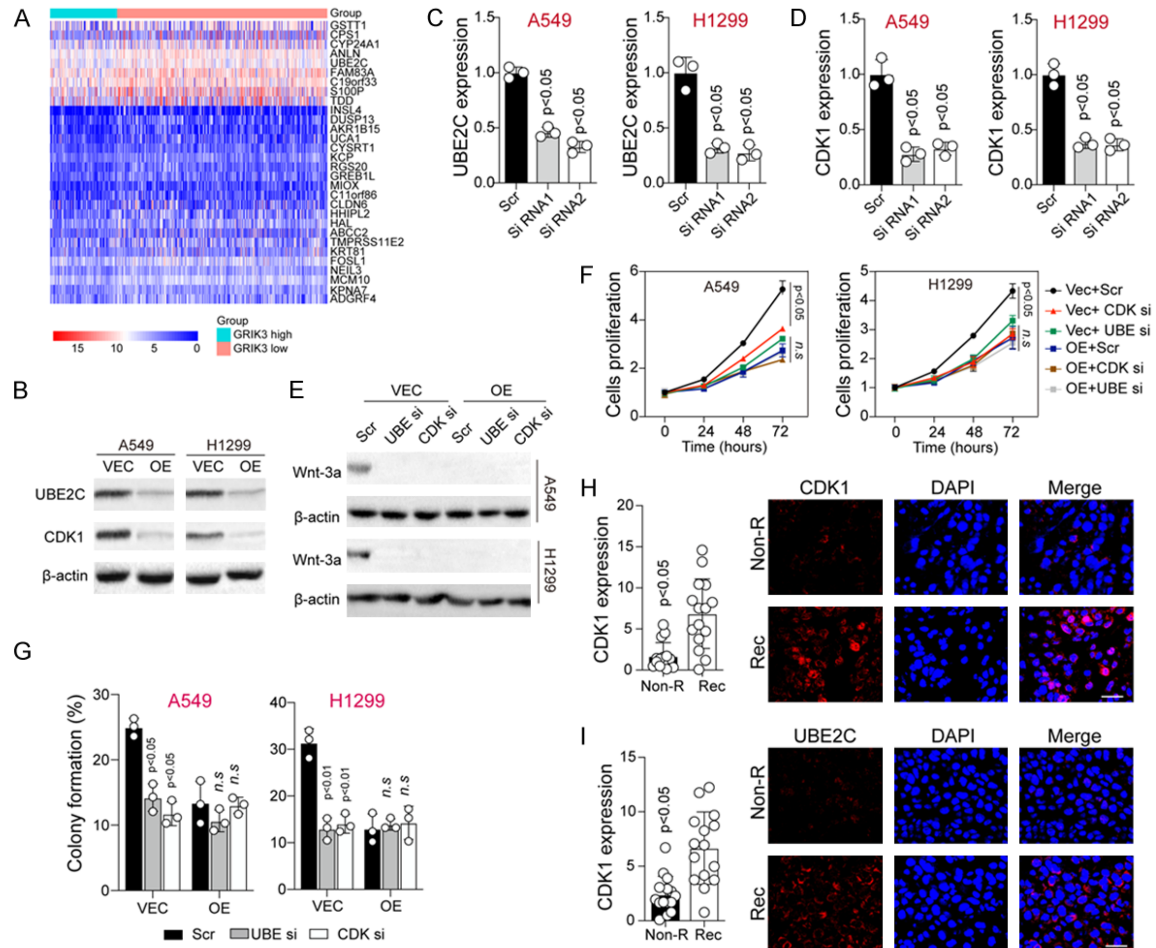

Figure 3. The deficiency of GRIK3 contributed to the activation of UBE2C/CDK1/Wnt signaling pathways. A. The top 30 downregulated genes in the high GRIK3 group (as compared to the low GRIK3 group) that were analyzed using the TCGA database. B. Western blotting of UBE2C and CDK1 in vector cells and A549/NCI-H1299 cells with GRIK3 overexpression. C. The relative expression of UBE2C in A549/NCI-H1299 cells treated with scramble siRNA, UBE2C siRNA #1 and 2, as determined by quantified PCR. D. The relative expression of CDK1 in A549/NCI-H1299 cells treated with scramble siRNA, CDK1 siRNA #1 and 2, as determined by quantified PCR. E. Western blotting of Wnt-3a in vector or GRIK3 overexpressed A549/NCI-H1299 cells treated with scramble siRNA, CDK1 siRNA, and UBE2C siRNA. F. The relative cell proliferation of vector cells and A549/NCI-H1299 cells with GRIK3 overexpression that were treated with scramble siRNA, CDK1 siRNA and UBE2C siRNA. G. The colony formation capability of vector cells and A549/NCI-H1299 cells with GRIK3 overexpression that were treated with scramble siRNA, CDK1 siRNA and UBE2C siRNA. H and I. Immunofluorescence of CDK1 and UBE2C in NSCLC tissues from recurrent (n=15) and non-recurrent (n=15) patients. The scale bar was 50 μm. The protein expression intensity was calculated.

48 hours (**Figure 3C** and **3D**). Consistent with our hypothesis, the silencing of UBE2C or CDK1 inhibited Wnt-3a expression in A549/NCI0H1299 cells, and limited influenced was found in GRIK3 overexpressed group (**Figure 3E**). This prompted us to suggest that GRIK3 suppressed Wnt signaling through the down-regulation of the UBE2C/CDK1 axis. Subsequently, we further evaluated cell proliferation and colony formation in UBE2C or CDK1 silenced A549/NCI-H1299 cells. Accordingly, UBE2C or CDK1 silencing led to the reduced capability of cell proliferation and colony formation in A549/NCI-H1299 cells. However, GRIK3 overexpression failed to suppress cell proliferation (**Figure 3F**) and colony formation (**Figure 3G**) when UBE2C or CDK1 was silenced, which suggested that GRIK3 inhibited NSCLC progression in a UBE2C/CDK1-dependent manner.

To further validate our results, we overexpressed CDK1 and UBE2C in GRIK3-overexpressed A549/H1299 cells (Figure S1B).

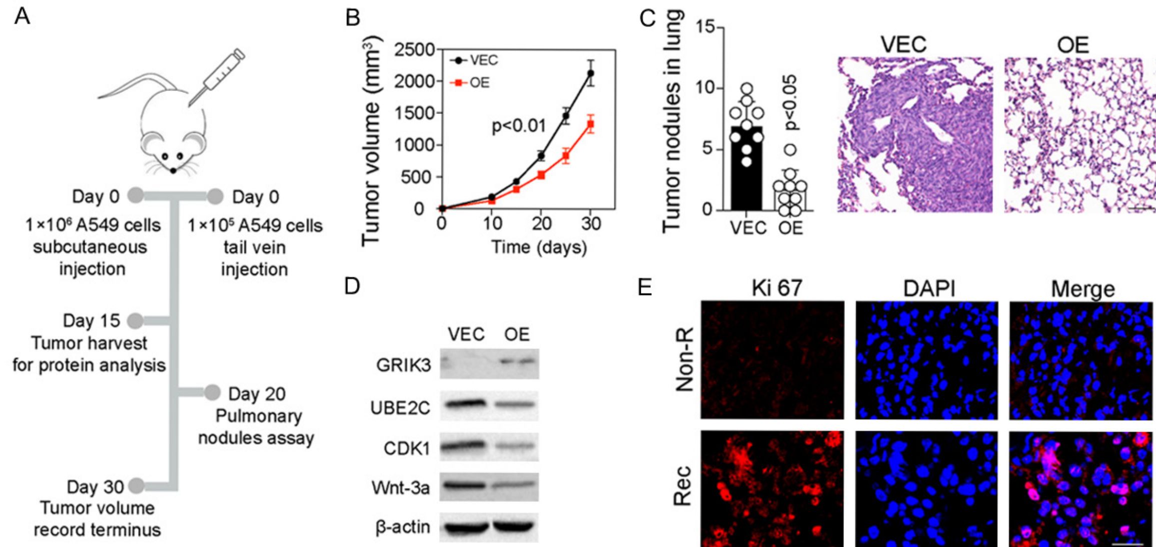

Figure 4. GRIK3 expression modulated the tumor progression of NSCLC *in vivo*. A. Mice were subcutaneously injected with 1×10^6 vector cells and A549 cells with GRIK3 overexpression for a tumor growth assay. The mice were injected with 1×10^5 vector cells and A549 cells with GRIK3 overexpression by the method of a tail vein injection. The lung tissues were collected on day 20 for conducting the metastatic tumor nodules assay. B. The tumor volume of subcutaneous vector cells and A549 mice with GRIK3 overexpression. C. The metastatic tumor nodules in lung tissues from mice were injected with 1×10^5 vector cells and A549 cells with GRIK3 overexpression by the method of tail vein injection (day 20). The scale bar was 200 μm for H&E staining. D. Western blotting of GRIK3, UBE2C, CDK1, and Wnt-3a in tumor tissues subcutaneous vector and A549 mice with GRIK3 overexpression. E. Immunostaining of Ki67 in tumor tissues subcutaneous vector and A549 mice with GRIK3 overexpression. The scale bar was 50 μm.

Intriguingly, overexpression of CDK1 and UBE2C upregulated the expression of Wnt3a (Figure S1C), enhanced cell proliferation (Figure S1D) and colony formation (Figure S1E) in GRIK3-overexpressed A549/H1299 cells. To elucidate the influence of the UBE2C/CDK1 axis on NSCLC progression, the expression of BUE2C and CDK1 was reanalyzed in clinical specimens from non-recurrent and recurrent groups. In agreement with our previous results, patients in the recurrent group exhibited a remarkable increase in UBE2C and CDK1 expression (**Figure 3H** and **3I**). These results indicated that GRIK3 downregulated Wnt signaling, and suppressed NSCLC progression through the UBE2C/CDK1 axis.

GRIK3 expression modulated NSCLC tumor progression in vivo

Based on the *in vitro* observation that GRIK3 downregulated UBE2C/CDK1/Wnt signaling to suppress NSCLC progression, we became interested in investigating the influence of GRIK3 on NSCLC development *in vivo*. To this end, we injected 1×10^6 vector and A549 cells with GRIK3 overexpression into immunodeficient mice, to establish a subcutaneous A549 mice model (**Figure 4A**). Accordingly, we found that the growth of tumors was slower in A549 cells with GRIK3 overexpression as compared to the vector group (**Figure 4B**). Subsequently, for further assessment of the tumorigenic and metastatic potential of GRIK3 overexpression cells, we injected 1×10^5 vector and A549 cells with GRIK3 overexpression into immunodeficient mice by the method of tail vein injection. After 20 days, the mice were sacrificed to perform a lung tumor nodules formation assay. In line with the result in tumor growth, A549 cells with GRIK3 overexpression demonstrated reduced tumorigenic and metastatic potential (**Figure 4C**). Meanwhile, the protein level of GRIK3, UBE2C, CDK1, and Wnt-3a were suppressed in the tumor tissues with GRIK3 overexpression, as determined by western blotting. Notably, this was in line with our results *in vitro* (**Figure 4D**). Additionally, immunostaining of Ki67, a proliferative marker in tumor tissues, was performed in vector and GRIK3 overexpression tumor tissues. This further provided evidence that GRIK3-suppressed expression of Ki67 and cell proliferation in tumor tissues (**Figure 4E**). Our findings demonstrate that GRIK3 functions

as an essential tumor suppressor for inhibiting NSCLC progression.

Discussion

Growing evidence suggests that the iGluRs family plays a critical role in the central nervous system and neurologic tumors [15-18], but its impact on solid tumor development remains unclear. Among the members of the iGluRs family, GRIK3 is a key participant in the neuroactive ligand receptor interaction pathway [19]. Previous studies have shown that other members of the GRIK family, such as GRIK1 and GRIK2, regulate cellular proliferation, migration, and epithelial-mesenchymal transition in malignancies [20]. For example, GRIK1 has been identified as a novel susceptibility gene for HBV-related hepatocellular carcinoma [21], while hypermethylation of the GRIK2 gene promoter inhibits its expression and reduces cell migration in gastric cancers [22]. However, our current understanding of GRIK3 has been limited, and it was unclear whether GRIK3 could modulate tumor phenotypes. Although a few studies have reported that the methylated GRIK3 gene is present in all stages of lung adenocarcinoma [10], the cellular function associated with epigenetic modification and changes in downstream signaling networks is unclear. In our study, we provide strong evidence that GRIK3 is downregulated in NSCLC and closely correlated with clinicopathological stages, tumor recurrence, and long-term survival of NSCLC patients. We further demonstrate that a decrease in GRIK3 expression can promote tumor growth and metastasis in vitro and in vivo. Our findings underscore the clinical significance of GRIK3 and provide a potential prognostic biomarker for NSCLC patients.

To explore the underlying mechanism of down-regulated GRIK3 in NSCLC progression, we systematically compared the differentially expressed genes between high and low GRIK3 groups. Additionally, we performed a KEGG analysis to determine the signaling pathways involved. Previous studies have indicated that GRIK3 could facilitate the epithelial-mesenchymal transition (EMT) process by inhibiting the epithelial markers, and increasing mesenchymal markers such as Zinc Finger E-Box Binding Homeobox 1 (ZEB1) and β-catenin in breast cancer [13]. This modulation might be relevant to the processes by which cancer cells enter the vascular system and settle in the lymph nodes and distant tumor sites. Importantly, our study is the first to report that down-regulation of GRIK3 could promote cell proliferation and migration in NSCLC by activating Wnt/β-catenin signaling, which is a commonly observed signaling abnormality associated with EMT and cancer metastasis. However, only a few studies have investigated the specific interaction between GRIK3 and Wnt/β-catenin signaling in NSCLC. Our study has provided initial evidence that GRIK3 can inhibit the UBE2C/CDK1 axis, which can subsequently mediate β-catenin activity. The down-regulation of GRIK3 can reverse this inhibition and lead to downstream activation of Wnt/β-catenin signaling in NSCLC progression.

It is well established that UBE2C is a member of the E2 ubiquitin-conjugating enzyme family, which plays a critical role in the ubiquitination system [23]. It has been shown that UBE2C expression has a strong correlation with other proto-oncogenes, particularly cell cycle-associated genes [24, 25]. These include members of the kinesin family (KIF20A, KIF18B, KIFC1, and KIF4A), cell division cycle 20 (CDC20), CDK1, and cyclin B1 (CCNB1), and the cell adhesion molecule complex (TROAP) [26, 27]. Our results consistently demonstrated that the upregulation of UBE2C promotes the expression and activation of CDK1, leading to the activation of Wnt/β-catenin signaling and uncontrolled cell proliferation and migration in NSCLC. Furthermore, our study was the first to report that GRIK3 inhibits the UBE2C-associated modification of cellular function and elucidated the clinical significance of the UBE2C/CDK1 axis in NSCLC patients. These findings suggest the possibility of developing innovative therapeutic targets for the treatment of NSCLC.

Based on our findings, we preliminary indicated the suppressive effect of GRIK3 in NSCLC progression and elucidated the down-regulated GRIK3 reversely increased migratory and invasiveness by activating UBE2C/CDK1/Wnt/β-catenin signaling. To do this, we established GRIK3-overexpressed cell lines and mice, and observed decreased tumor growth and metastasis *in vitro* and *in vivo*. Notably, our results were supported by RNA-seq and survival data from TCGA as well as findings from pathological tumor tissues of clinical NSCLC patients. However, our study has some limitations. The

analyzed public databases were insufficient, and the number of clinical pathological tissue samples was limited, which restricts the clinical relevance of our findings. Nonetheless, these preliminary data could assist ongoing research. Although the tumor-suppressive role of GRIK3 in NSCLC has been investigated in previous studies to a certain extent, further research is needed to understand the mechanisms underlying its effects and the GRIK3-centric protein interaction networks. It is yet to be determined how GRIK3, a membrane protein, inhibits the function of UBE2C, a member of the cellular ubiquitination system. Additionally, the UBE2C-associated protein network is complex and has strong correlations with some tumor suppressors [28-30]. Future functional studies are required to clarify the role of UBE2C in cancer, investigate its associated pathways, and explore potential therapeutic targets for cancer treatment.

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Further, we have obtained informed consent to conduct experiments on human subjects.

Disclosure of conflict of interest

None.

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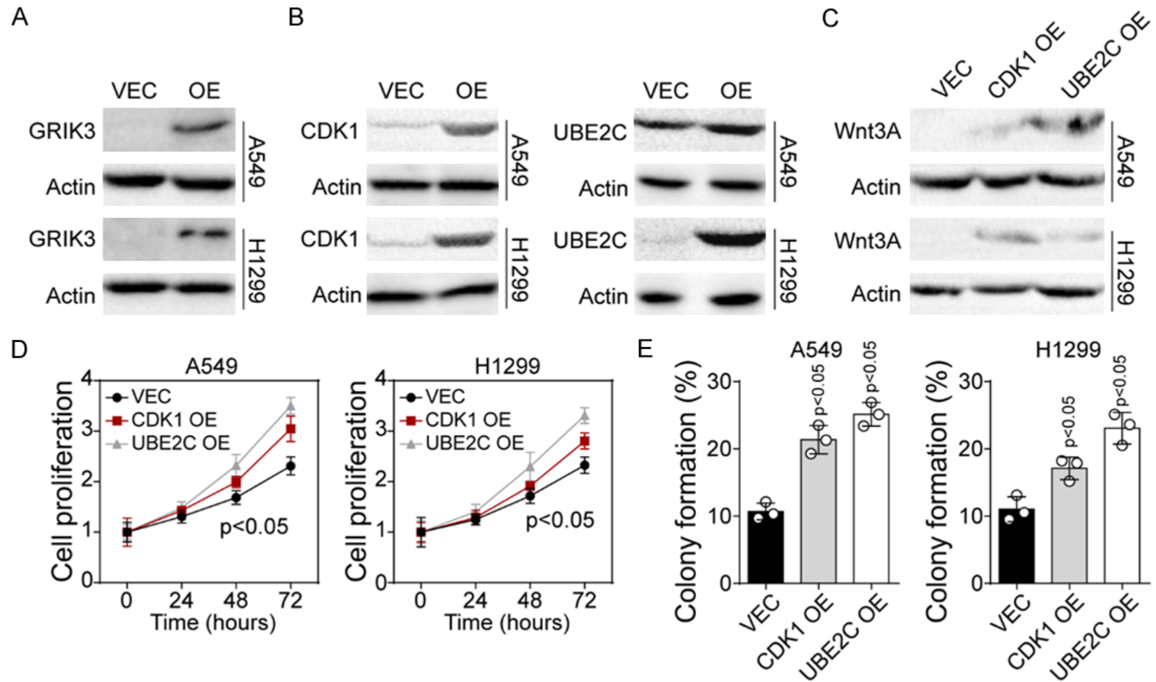

Figure S1. (A) Western blotting of GRIK3 in vector or GRIK3 overexpressed A549/H1299 cells. (B) CDK1 and UBE2C were overexpressed in GRIK3 overexpressed A549/H1299 cells respectively, and the protein level of CDK1 and UBE2C were determined by western blotting. (C-E) CDK1 or UBE2C was overexpressed in GRIK3 overexpressed A549/H1299 cells. Then the Wnt3A expression level (C), cell proliferation (D) and colony formation (E) capability were determined.