Research Square

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L-Theanine inhibits cancer stem cell-mediated chemoresistance in lung cancer by regulating STAT3/NOTCH1-BMAL1 signaling

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Research Article

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Abstract

Cancer stem cells play crucial roles in the development of cancer chemoresistance. L-Theanine, a nonproteinogenic amino acid derived from green tea, is gaining more and more attentions in reversing cancer drug resistance. However, its roles in development of lung cancer chemoresistance is still unknown. To investigate the effects of L-Theanine on chemoresistance and explore its underlying mechanism in lung cancer, we performed a panel of experiments in vitro combined with RNA-seq analysis and demonstrated L-Theanine improved the chemoresistance to cisplatin (cis-diamminedichloroplatinum; DDP) and inhibited stemness of DDP-resistant lung cancer cells but not non-resistant lung cancer cells and STAT3/NOTCH1 signaling was a potential dominant process involved in L-Theanine improving chemoresistance in DDP-resistant lung cancer. Mechanistically, L-Theanine impedes DDP-resistant lung cancer cells migration and activation via regulating the expression of STAT3/NOTCH1/BMAL1 signaling-induced stemness markers, reducing the migration and proliferation of DDP-resistant lung cancer cells as well as inhibiting drug resistance-related genes expression in DDP-resistant lung cancer cells. In addition, combination of L-Theanine and Stat3 blockade improved synergistically the chemoresistance in DDP-resistant lung cancer. In summary, L-Theanine improved chemoresistance in chemoresistant lung cancer through regulating STAT3/NOTCH1/BMAL1 signaling, reducing stemness and finally inhibiting the migration of DDP-resistant lung cancer cells. The finding might provide the research evidence for therapeutic options of reversing chemoresistance in lung cancer.

Introduction

Cancers are considered to be heterogeneous entities, and it is the presence of cancer heterogeneity that makes chemotherapy not able to completely remove the cancer, and the cause of cancer heterogeneity tends to be the presence of cancer stemness [1]. Cancer stemness is believed to be the initiation of chemotherapy resistance [2]. Conventional chemotherapy kills cancer cells but has little effect on cancer stem cells, and recurrent cancers tend to be more drug-resistant [3]. Therefore, investigating the mechanism of chemoresistance CSCs will help to improve the conventional chemotherapy regimen and enhance the overall efficacy of chemotherapy.

Non-small cell lung cancer (NSCLC) accounts for about 80–85% of lung cancers and is the leading cause of cancer deaths worldwide. Although EGFR, ALK, ROS1, KRAS, c-MET, RET, NTRK, BRAF and HER2 mutation-positive patients are suitable for molecularly targeted therapies, resistance to targeted therapies inevitably occurs in these patients over time[4]. After targeted therapies, patients usually receive systemic chemotherapy (e.g., platinum-based chemotherapy) with or without immunotherapy with immune checkpoint inhibitors (ICIs). Cisplatin is one of the most effective chemotherapeutic agents for the treatment of human cancers, including NSCLC. Unfortunately, many lung cancer patients develop resistance to cisplatin-based therapy [5, 6]. Therefore, understanding the underlying mechanisms of action of platinum-based compounds is necessary to overcome the barriers to the clinical use of platinum-based compounds in lung cancer patients. Currently, platinum drug resistance mechanisms include the following aspects: reduced intracellular platinum accumulation, inactivation of intracellular

platinum, enhanced DNA damage repair, enhanced inhibition of apoptosis in cancer cells, etc [7, 8]. However, of note, as mentioned above, current chemotherapeutic agents can kill most cancer cells, but their ability to kill resting CSCs is extremely limited, hence CSCs have been recognized as one of the more fundamental causes of chemoresistance in cancers. Identifying and effectively inhibiting CSCs may be the key to reversing chemoresistance in lung cancer.

In recent years, it has been found that L-theanine, one of the unique chemical components of tea, has not only anti-cancer effects, but also has bioregulatory effects on certain anti-cancer drugs [9, 10]. However, L-theanine's roles in chemotherapy resistance in cancers, especially lung cancer, are understudied. In the study, we demonstrated L-Theanine relieved the chemoresistance to Cisplatin; mechanistically, L-Theanine inhibited lung cancer stem cells migration and activation through regulating Notch1/STAT3/BMAL1 signaling and finally overcome cisplatin resistance in lung cancer.

Materials and Methods

Cell culture

A549, A549/DDP, NCI-H446 and NCI-H446/DDP cells used in this study were purchased from the National Authorized Cell Culture Preservation Center (Chinese Academy of Sciences, Shanghai, China). Cell culture was performed according to the manufacturer's protocol. Cells were cultured in DMEM medium (HyClone, Thermo Fisher Scientific, Waltham, USA) supplemented with 10% (v/v) FBS, 100 units/mL penicillin G, 100 μg/mL streptomycin sulfate, and 2 mM L-glutamine. All cells were cultured at 37°C in a humidified incubator with 5% (v/v) carbon dioxide in air.

Cytotoxity assay

The cells were cultured in a 96-well plate, with a density of 1×10^3 cells/well. After treatment for the desired time, CCK-8 kit (Dojindo, Japan) was used to detect the cell viability according to manufacturer's instructions. Multiskan GO microplate reader (Thermo Scientific, Waltham, MA, USA) was used to count the cells. Data were averaged from at leastthree independent experiments and are expressed as means ± SD.

Transwell assay

Transfected cells or treated cells were plated on the upper part of a transwell chamber in serum-free media with 10% fetal bovine serum was added in the lower part of the chamber as chemoattractant. After 6 h or 12 h of incubation at 37°C, cells inside the upper part were removed. Migrated cells on the lower membrane surface were analyzed by crystal violet staining.

Tumorsphere formation assay

Tumor sphere formation assay was performed as previously described. Lung cancer cells were seeded into ultra-low attachment 24-well plates (Corning) in DMEM medium supplemented with 20 ng/mL epidermal growth factor (EGF), 10 ng/mL basic fibroblast growth factor (bFGF), 5 mg/mL insulin and 2%

B27 at 37°C in a humidified atmosphere of 95% air and 5% CO2. Half medium with additions were renewed every other day. Tumorspheres were visualized and calculated by a microscope.

RNA-seq and quantitative PCR analysis

RNA-seq libraries were established with an Illumina TruSeq RNA sample prep kit and sequenced using Illumina HiSeq 4000 with 150-bp paired-end sequencing strategy. Expression analysis was performed using HISAT2 and DESeq2, and differentially expressed genes were determined with the cutoff fold change ≥ 1.5 and p-value ≤ 0.05. The KEGG enriched pathway was determined according to ErichR, the web-based software for gene set enrichment analysis.

Western blotting

Total protein was extracted from cultured cells using RIPA buffer and protease inhibitor cocktail, and protein concentration was determined by Pierce BCA proteometric assay (Thermo Fisher Scientific, MA, USA). Aliquots of protein samples were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, MA, USA). The following antibodies were used: rabbit polyclonal anti-ALDH1 antibody (1:1500; Santa Cruz, USA), rabbit polyclonal anti-ABCG2 antibody (1: 1000; Santa Cruz, USA), rabbit polyclonal anti-Nanog antibody (1: 1500; Santa Cruz, USA), mouse monoclonal anti-NOTCH1 antibody (1: 500, ThermoFisher, USA), rabbit polyclonal anti-phospho-STAT3 antibody (1: 500, ThermoFisher, USA), Rabbit polyclonal anti-STAT3 antibody (1: 500, Abcam, USA), Rabbit polyclonal anti-BMAL1 antibody (1: 500, Abcam, USA), Mouse monoclonal anti-β-actin antibody (1: 1000, Santa Cruz, USA).

Lentivirus-mediated shRNA transfection

The lentivirus-mediated BMAL1 knockdown and overexpression system and corresponding controls were constructed by GeneCreate (Wuhan, China). Transfection was performed using the transfection reagent Endo-FectinTM Max transfection (GeneCopoeia) according to the manufacturer's protocol. 50 nM RNA oligonucleotides and 10 μl Endo-FectinTM Max were diluted in 250 μl Opti-MEM (Thermo Fisher Scientific, Inc.) Then they were mixed and incubated at room temperature for 20 min to form complexes. Cells (1×105) were incubated with the complex for 6 hours, then cells were kept in fresh medium and harvested at the indicated times after transfection for real-time PCR analysis and Western blotting.

Statistical analysis

SPSS 24.0 software was used for the statistical analysis. Continuous variables were expressed as mean ± SEM and compared through Wilcoxon test. Categorized variables were presented by frequency (n) and proportion (%), and then compared through ANOVA. Independent t test was used for the comparisons of the data between groups. Both Cox proportional hazard model and Log-rank test were applied to survival analyses. $P < 0.05$ was considered statistically significant.

Results

L-Theanine relieved the chemoresistance to cisplatin in resistant lung cancer cells

In order to investigate the effect of L-Theanine on the chemosensitivity of lung cancer cells, we examined the changes in the sensitivity of lung cancer cells A549 and NCI-H446 to DDP after L-Theanine treatment. The results showed that L-Theanine did not cause a significant enhancement in the sensitivity of lung cancer cells to DDP treatment (Fig. 1A-B). However, it is noteworthy that for DDP-resistant strains (A549/DDP, NCI-H446/DDP), the resistance index of cells to DDP was significantly reduced after L-Theanine treatment (Fig. 1C-D).

L-Theanine inhibited the stemness of chemoresistance lung cancer cells

As mentioned previously, there is a close relationship between cancer stemness and platinum-based chemotherapy resistance. In order to clarify whether the effect of L-Theanine on drug resistance in lung cancer is related to cancer stemness, we first examined the changes in the clone-forming ability of L-Theanine-treated lung cancer drug-resistant cells. The results showed that L-Theanine significantly inhibited the clone formation ability of DDP-resistant lung cancer cells, however, it had no significant effect on the clone formation ability of non-drug-resistant cells (Fig. 2A-D). Subsequently, we examined the effect of the expression of stemness markers in drug-resistant and non-drug-resistant lung cancer before and after L-Theanine treatment; the results revealed that L-Theanine led to a decrease in the expression of stemness markers in drug-resistant lung cancer cells, whereas no significant decrease in non-drug-resistant lung cancer cells (Fig. 2E-H). In addition, L-Theanine also inhibited the invasion ability of drug-resistant lung cancer cells but not non-drug-resistant lung cancer cells (Fig. 2I-L). In summary, L-Theanine may be targeted to inhibit the stemness of drug-resistant lung cancer cells without significant effect on the stemness of non-drug-resistant cells, which will be helpful for personalized development of reversal strategies for chemotherapy-resistant lung cancer.

Transcriptomics analysis revealed STAT3/NOTCH1 signaling involved in L-Theanine relieving chemoresistance in lung cancer

In order to clarify the differences in the effects of L-Theanine on DDP-resistant and non-drug-resistant lung cancer cells and the intrinsic mechanisms, we performed a comparative analysis of the RNA-seq data of L-Theanine-treated drug-resistant lung cancer cells and non-drug-resistant lung cancer cells. For non-drug-resistant lung cancer cells, a total of 725 differentially expressed genes were identified between the L-Theanine-treated group and the control group, including 278 up-regulated genes and 447 down-regulated genes; for drug-resistant lung cancer cells, a total of 829 differentially expressed genes were identified between the L-Theanine-treated group and the control group, including 387 up-regulated genes

and 442 down-regulated genes; relative to non-drug-resistant, a total of 316 differentially expressed genes were identified in L-Theanine-treated drug-resistant lung cancer cells, of which 208 were up-regulated genes among which BMAL1 was significantly upregulated in L-Theanine-treated drug-resistant lung cancer cells, and 108 were down-regulated genes (all of which were used as the background for their respective controls). DEGs in L-Theanine-treated drug-resistant lung cancer cells relative to non-drug-resistant lung cancer cells were statistically enriched in molecular Signature Data Base (mSigDb) markers and in the Gene Ontology of Biological Processes (GO), they are statistically enriched for genes associated with biosynthesis (fatty acids, glycosylphosphatidylinosital, etc), signaling transduction, transcription factors and virus infection; subsequent KEGG and Reactome enrichment analysis identified 239 genes enriched in cancer-related signaling pathways, including the STAT3/NOTCH1 signaling pathway and the IL-17 signaling pathway (Fig. 3A-C).

BMAL1 participated in the regulation of lung cancer cells stemness as the downstream of STAT3/NOTCH1 signaling

Based on the analysis of transcriptomics data, we further explored the effect of L-Theanine on STAT3/NOTCH1 signaling in lung cancer cells. The results showed that L-Theanine treatment resulted in a significant decrease in the levels of phosphorylated STAT3 and Notch1 in drug-resistant lung cancer cells, which was not observed in non-drug-resistant lung cancer cells (Fig. 4A-D). We also observed that BMAL1 expression was significantly reduced in L-Theanine-treated drug-resistant lung cancer cells, which was relatively not significantly altered in L-Theanine-treated non-drug-resistant lung cancer cells (Fig. 4E-H). It has been shown that in cervical cancer, STAT3/NOTCH1 signaling activation can upregulate BMAL1 expression [11]. To further clarify the regulation of BMAL1 expression by STAT3/NOTCH1 signaling in lung cancer cells, we treated drug-resistant lung cancer cells with Stattic, a specific inhibitor of STAT3, and found that BMAL1 protein expression was significantly reduced when phosphorylated STAT3 was significantly inhibited, suggesting that the expression of BMAL1 was affected by STAT3/NOTCH1 signaling regulation. In addition, we also found that the expression of cell stemness markers was significantly reduced after the effective knockdown of BMAL1 in drug-resistant lung cancer cells (Fig. 4I-L), and the clone formation and invasive migration ability of cells were also significantly inhibited (Fig. 4M-P). The above results suggest that L-Theanine can inhibit BMAL1 expression in drug-resistant lung cancer cells by regulating STAT3/NOTCH1 signaling, which in turn inhibits cancer stemness.

Combination of L-Theanine and STAT3 blockade improved synergistically the chemoresistance in lung cancer

Based on the above findings, we further explored the possibility of reversing drug resistance in lung cancer cells by combining L-Theanine with STAT3 blockade. First, we observed the changes in the cellular resistance index to DDP after L-Theanine combined with STAT3 blockade treatment of drug-resistant lung cancer cells. The results showed that the combination treatment synergistically reduced the resistance index of drug-resistant lung cancer cells relative to monotherapy (Fig. 5A-B). Second, we also found that L-Theanine combined with STAT3 blockade treatment significantly inhibited the clone formation ability

as well as the invasive migration ability of drug-resistant lung cancer cells (Fig. 5C-D). Finally, we further found that L-Theanine combined with STAT3 blockade treatment effectively reduced the expression levels of stemness markers in drug-resistant lung cancer cells (Fig. 5E-H). These results suggest that L-Theanine combined with STAT3 blockade synergistically inhibits the stemness of drug-resistant lung cancer cells and thus overcomes platinum-based chemotherapy resistance in lung cancer cells.

Discussion

Theanine is a special secondary metabolite of tea, which is one of the non-protein free amino acids unique to tea with the highest content. In recent years, with the maturity and application of modern biotechnology, the pharmacological mechanism of theanine has been widely studied [12]. At present, many studies at home and abroad have shown that theanine and its synthetic derivatives can induce apoptosis of cancer cells and inhibit their growth and invasion and metastasis, thus preventing and controlling cancers; moreover, theanine can also enhance the activity of certain anticancer drugs and have certain effects on alleviating the adverse reactions of anticancer drugs, which plays an important role in the development of cancer chemotherapy [13, 14]. Therefore, the multiple medicinal values of theanine have a broad application prospect in the prevention and treatment of cancers, and it is worthwhile to continue further exploration and research. In the study, we revealed for the first time that L-Theanine plays an important role in cisplatin-resistant lung cancer cells but not parental lung cancer cells through performing a serial of cell-based assays; mechanistically, L-Theanine was demonstrated to inhibit stemness of cisplatin-resistant lung cancer cells by regulating Notch1/STAT3/BMAL1 signaling. These findings suggest a potential value of L-Theanine in overcoming chemoresistance in lung cancer.

Platinum-based drugs have always been the mainstay of combinations in precision therapy and immunotherapy for many years, and one of the research mainstays on platinum-based drugs has been the resistance mechanisms, many of which have been extensively addressed in preclinical models and provided a guidance in the treatment of cancers [15]. Lung cancer is a malignant cancer with high morbidity and mortality worldwide. However, when the targeted therapeutic drugs appear resistant, platinum-based chemotherapy is still the most important treatment modality. Unfortunately, the clinic observations show that chemotherapy is more effective in its early application in lung cancer patients, but inevitably becomes resistant in the later stage. In these years, L-Theanine has been verified to suppress the proliferation and migration and induce apoptosis via inhibiting VEGFR/EGFR or NF-kB signaling in lung cancer [16–18]. However, it is still unknown about the association between L-Theanine and lung cancer chemotherapeutic resistance. Hence, in the study, we tried to address the issue and demonstrated that L-Theanine led to the significant decrease of the resistance index of DDP-resistant lung cancer cells (A549/DDP, NCI-H446/DDP); in contrast, IC50 of lung cancer cells without DDP-resistance (A549 and NCI-H446) was not significantly decreased after L-Theanine treatment. The result indicated that L-Theanine can only affect the resistance phenotype of DDP-resistant lung cancer cells, but not lung cancer cells without resistance, which suggesting the potential application of L-Theanine in lung cancer patients with chemoresistance not in lung cancer patients with early stage as well as the important value of L-Theanine in overcoming the lung cancer chemoresistance.

It has been recognized that inhibition of cancer stemness can reduce the drug resistance potential of cancer cells [19]; in fact, the evolution of cancer stemness and the development of drug resistance potential interact each other [20]. Intriguingly, we found L-Theanine can significantly decrease the expression of cancer stemness-related markers in DDP-resistant lung cancer cells (A549/DDP, NCI-H446/DDP) but not in lung cancer cells without DDP-resistance (A549 and NCI-H446). The finding indicates that L-Theanine-induced inhibition of cancer stemness may reduce the progression, but not the formation, of drug resistance in lung cancer cells, which will help to design a precise intervention strategy against cancer stemness for reversing platinum-based chemoresistance in lung cancer.

However, how is L-Theanine involved in the regulation of DDP-resistance in lung cancer? For the reason, we performed transcriptomics-based comparative analysis and found that, compared to in lung cancer cells without DDP-resistance, L-Theanine-mediated DEGs in DDP-resistant lung cancer cells were enriched in DNA damage, stemness regulation and ABC transporter etc. Drug resistance in cancers is involved in three main mechanisms depending on the means of treatment, including high expression of drug transporter, strong DNA repair ability and recruitment of a protective niche [21, 22]. Moreover, KEGG analysis on RNA-seq data showed the dominant pathway is STAT3/NOTCH1 signaling pathway. Recent studies have revealed that the aberrant activation of STAT3/Notch1 signaling play a critical role in the interaction between cancer stemness and therapy resistance [23, 24], targeting STAT3/Notch1 signaling may inhibit the development of cancer stemness and reverse platinum-based chemoresistance [25, 26]. Notably, in the study, L-Theanine inhibited significantly the activation of STAT3/Notch1 signaling in DDP-resistant lung cancer cells; meanwhile the specific inhibition of STAT3/Notch1 signaling decreased significantly the formation of cancerspheres, the migration and the resistance index of DDP-resistant lung cancer cells as well as reduced obviously the expression of BMAL1, a top DEG in the transcriptomics analysis above.

The BMAL1 gene, also known as the ARNTL gene, is a key component in the molecular circadian oscillator of mammalian life activity [27]. In recent years, a large number of studies have shown that the BMAL1 plays an important role in cancerogenesis, development, therapeutic efficacy and prognosis; however, the effect of BMAL1 gene on cancers is extremely complex, in different cancers, BMAL1 gene can affect cancer development through different mechanisms, even in the same type of cancer, different microenvironments may make BMAL1 gene play different roles [28, 29]. Hence, further elucidation of the relationship between the BMAL1 and cancers may lead to a deeper understanding of the mechanisms of cancer genesis, dissemination and metastasis, and provide theoretical bases for the development of new models of clinical oncology diagnosis and treatment. Recent studies have revealed that BMAL1 can promote chemoresistance of various cancers including colon cancer, pancreatic cancer, adrenocortical carcinoma and etc.[30–32]. Importantly, based on the studies above indicating BMAL1 as a driver factor of chemoresistance, it was surprising that L-Theanine apparently inhibited the expression of BMAL1 in DDP-resistant lung cancer cells in the present work; furthermore, our results also suggested BMAL1 knockdown downregulated the expression of lung cancer stemness-related markers and inhibited the formation of tumorspheres, consistent with some studies [33, 34]. Altogether, a novel L-Theanine-STAT3/Notch1-BMAL1 regulatory axis, which connects chemoresistance with cancer stemness, was

established. The potential application value of the regulatory axis was explored and identified through using the combination of L-Theanine and STAT3 blockade in DDP-resistant lung cancer cells, which showing L-Theanine combined with STAT3 blockade synergistically inhibits the stemness of drug-resistant lung cancer cells and thus overcomes platinum-based chemotherapy resistance in lung cancer cells.

In summary, our study demonstrated that L-Theanine impede the progression, not the formation, of chemoresistance in lung cancer and inhibited the stemness by regulating STAT3/Notch1-BMAL1 axis. The finding might provide the research evidence for making a novel therapeutic strategy of reversing chemoresistance in lung cancer in the future.

Declarations

Author Contributions

W.J. and C.Z. conceived and designed the study, W.J., L.S., H.Y. and Z.D., M.L. contributed to carry out the experiments and W.J., L.S., H.Y. contributed to data analysis. W.J., L.S., H.Y. and Z.D., M.L. wrote the manuscript. C.Z. supervised the study. All authors have agreed on the journal to which the article has been submitted and agree to be accountable for all aspects of the work.

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Data Availability

Not applicable.

Conflict of interest

The authors have declared no conflict of interest.

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Figures

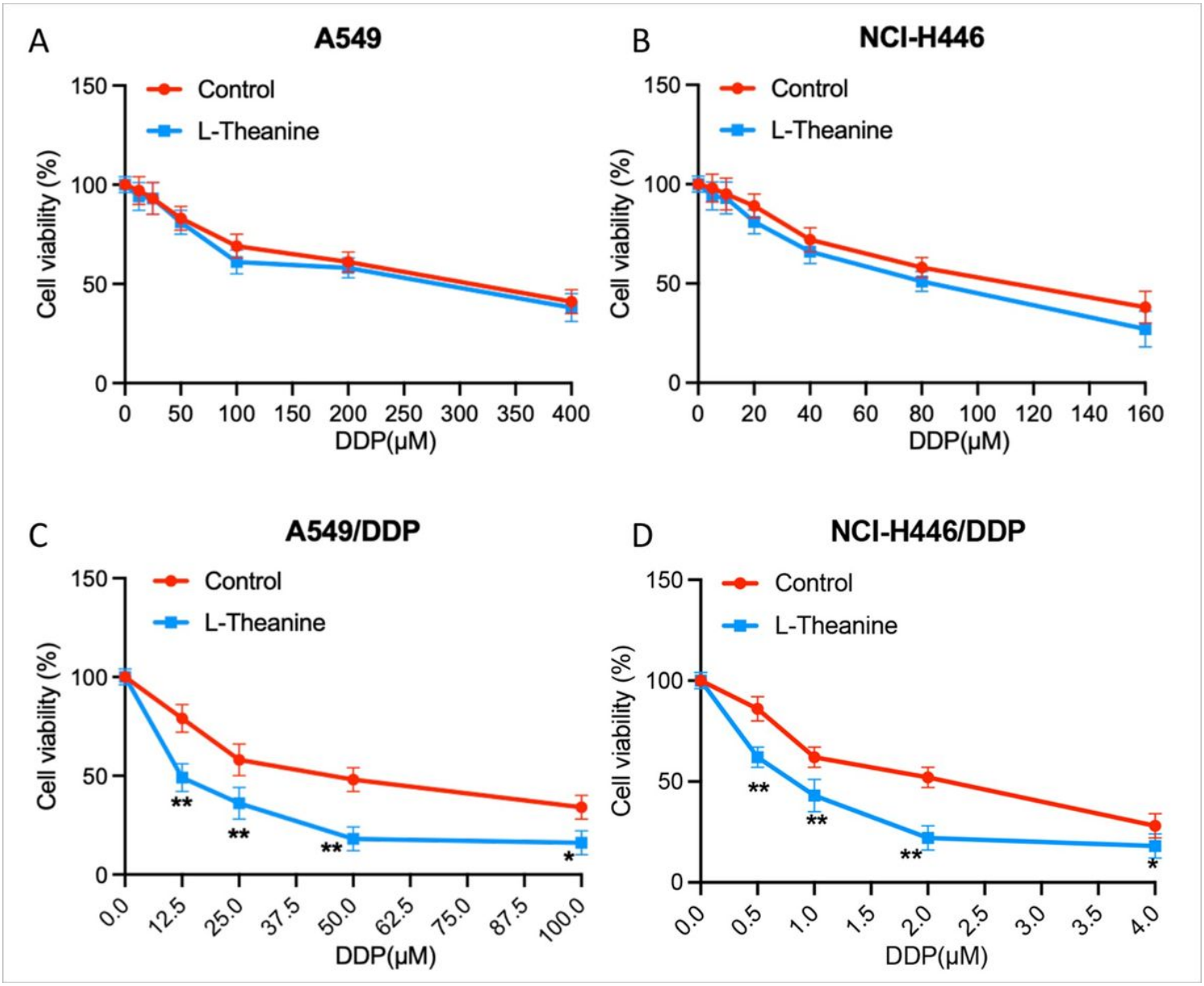


Figure 1

L-Theanine relieving the chemoresistance to cisplatin in resistant lung cancer cells. A549, A549/DDP, NCI-H446 and NCI-H446/DDP cells was cultured according to the manufacturer's protocol and were treated with increasing concentrations of cisplatin (0-400 μM for A549 and A549/DDP; 0-160 μM for NCI-H446 and NCI-H446/DDP). CCK-8 analysis showed the viability of the above cells following 48h cisplatin (DDP) treatment. Data are representative of three independent experiments and are presented as Mean ± SEM. *indicated significant difference. *p < 0.05, **p < 0.01.

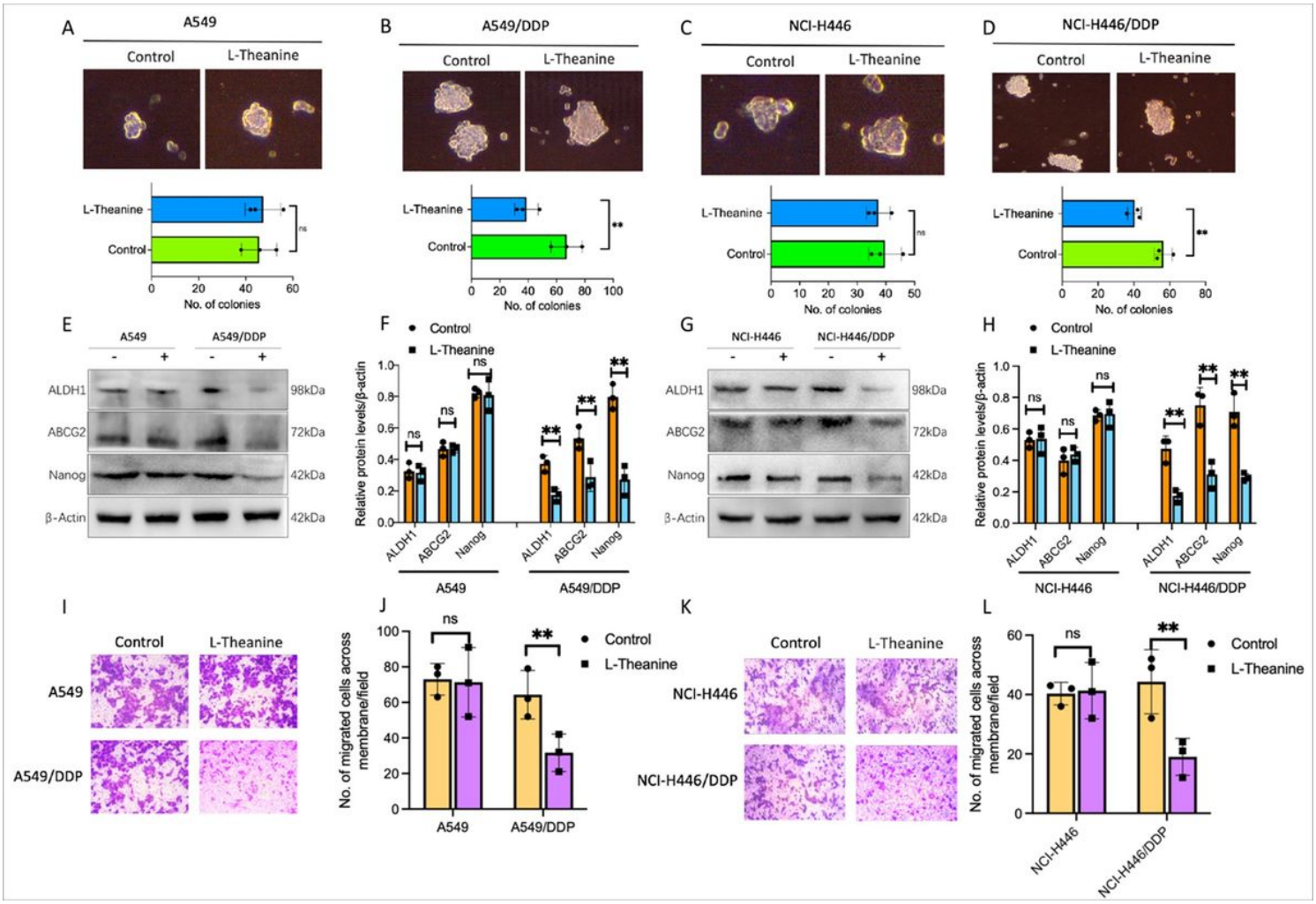


Figure 2

L-Theanine inhibiting the stemness of chemoresistance lung cancer cells. Tumorsphere formation assay was performed in A549, A549/DDP, NCI-H446 and NCI-H446/DDP cells with or without L-Theanine treatment for 14 days. The number of tumorspheres means colonies per 5000 cells (A-D). The protein levels of stemness-related factors including ALDH1, ABCG2 and Nanog in A549, A549/DDP, NCI-H446 and NCI-H446/DDP cells with or without L-Theanine treatment were analyzed by a western blotting assay (E-H). The indicated cells were treated with DDP for 14 days. Images and quantification of transwell migration assays; cells were stained with crystal violet (I, K). The bar graphs show the statistical analysis of the number of cells cross membranes (J, L). Error bars indicate SEM; n=3. *P<0.05.

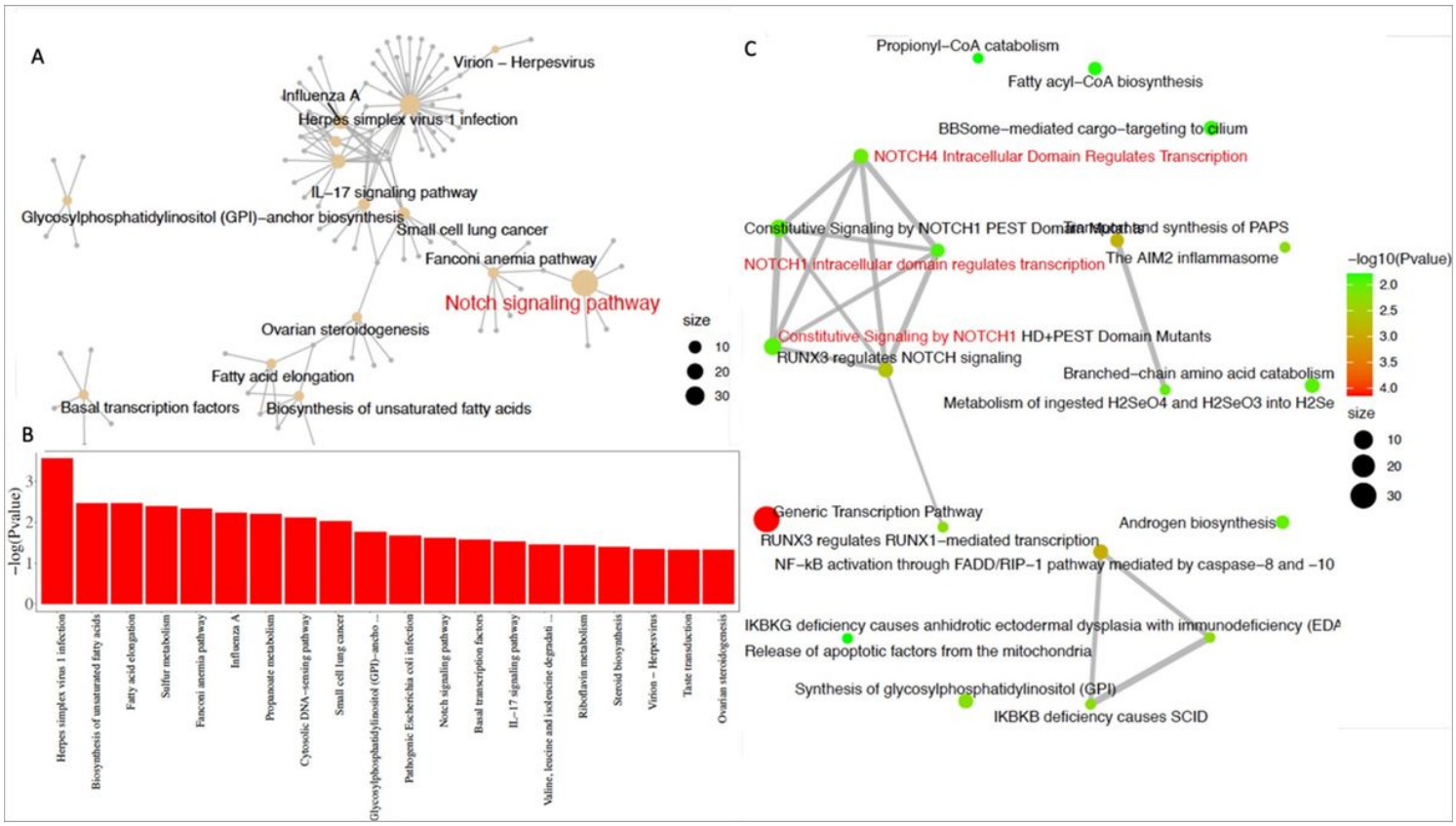


Figure 3

Involvement of STAT3/NOTCH1 signaling in L-Theanine relieving chemoresistance in lung cancer. Different expressed genes in L-Theanine-treated drug-resistant lung cancer cells relative to non-drug-resistant lung cancer cells were statistically enriched in molecular Signature Data Base (mSigDb) markers and in the Gene Ontology of Biological Processes (GO)(A), they are statistically enriched for genes associated with biosynthesis (fatty acids, glycosylphosphatidylinosital, etc), signaling transduction, transcription factors and virus infection (B); subsequent KEGG and Reactome enrichment analysis identified 239 genes enriched in cancer-related signaling pathways, including the STAT3/NOTCH1 signaling pathway and the IL-17 signaling pathway (C).

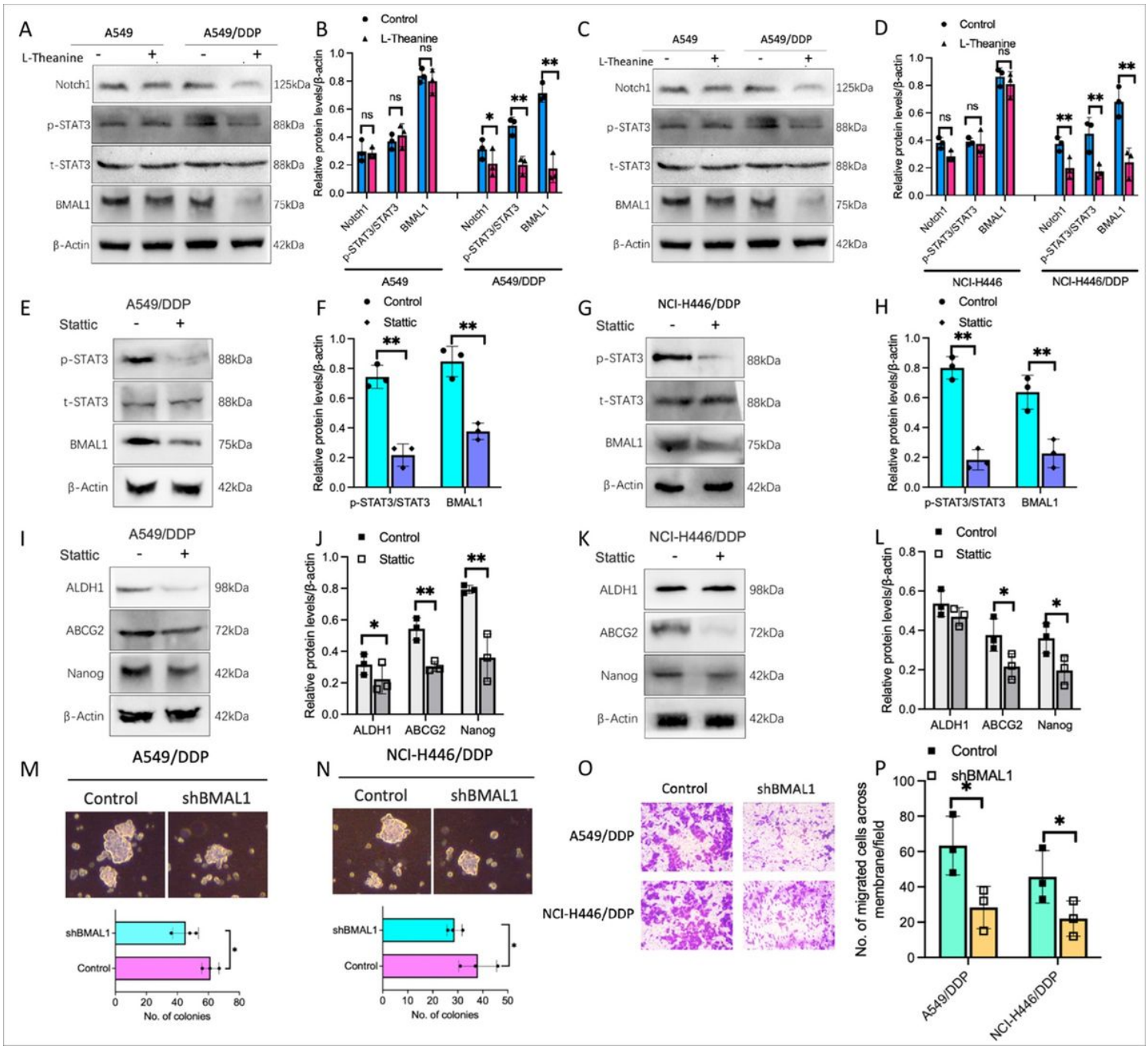


Figure 4

Involvement of BMAL1 in the regulation of lung cancer cells stemness as the downstream of STAT3/NOTCH1 signaling. The protein levels of Notch1, BMAL1 and p-STAT3/t-STAT3 in A549, A549/DDP, NCI-H446 and NCI-H446/DDP cells with or without L-Theanine treatment were analyzed by a western blotting assay (A-D). Moreover, The protein levels of BMAL1, p-STAT3/t-STAT3 and stemness-related factors including ALDH1, ABCG2 and Nanog in A549, A549/DDP, NCI-H446 and NCI-H446/DDP cells with or without stattic treatment were analyzed by a western blotting assay (E-L). Tumorsphere formation assay was performed in A549/DDP and NCI-H446/DDP cells with or without shBMAL1 knockdown for 14 days. The number of tumorspheres means colonies per 5000 cells (M-N). In addition,

images and quantification of transwell migration assays were presented in A549/DDP and NCI-H446/DDP cells with or without shBMAL1 knockdown (I-L). Error bars indicate SEM; n=3. *P<0.05.

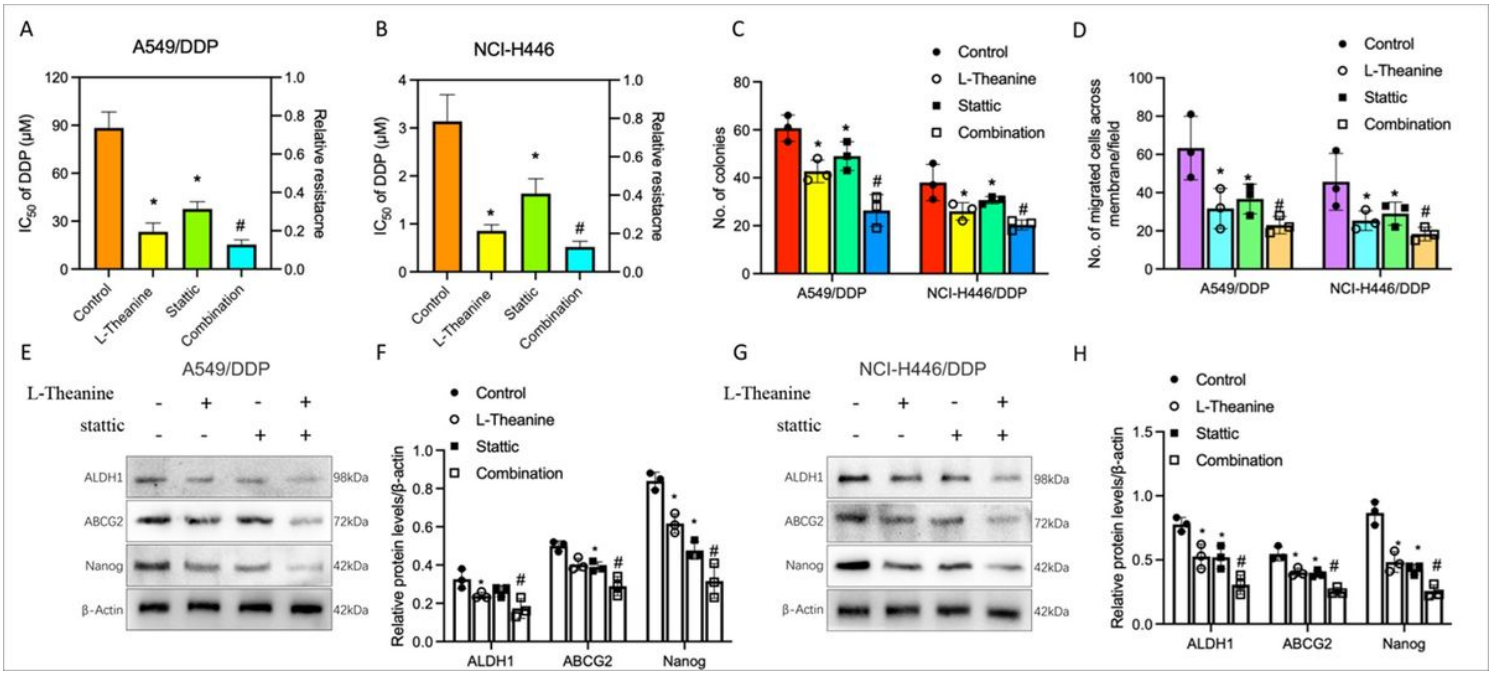


Figure 5

Combination of L-Theanine and STAT3 blockade improved synergistically the chemoresistance in lung cancer. The changes in the cellular resistance index to DDP after L-Theanine combined with STAT3 blockade treatment of A549/DDP and NCI-H446/DDP cells (A-B). Moreover, number of colonies per 5000 A549/DDP and NCI-H446/DDP cells was quantified (C); meanwhile, number of migrated cells (A549/DDP and NCI-H446/DDP cells) was calculated (D). In addition, The protein levels of stemness-related factors including ALDH1, ABCG2 and Nanog in A549, A549/DDP, NCI-H446 and NCI-H446/DDP cells with or without combination treatment were analyzed by a western blotting assay (E-H). Error bars indicate SEM; n=3. *P<0.05.