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High expression of NFAT2 contributes to carboplatin resistance in lung cancer

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ABSTRACT

Carboplatin is a platinum-based chemotherapy drug in lung cancer treatment. However, its efficacy is frequently limited by intrinsic and acquired drug resistance. Recently, nucleus factor of activated T cells, cytoplasmic 1 (NFAT2) has been recognized as an oncogene and involved in disease progression and drug resistance in various cancers. In the current study, we found that overexpression of NFAT2 was associated with poor prognosis in lung cancer patients, and is observed in a carboplatin resistant lung cancer cell line, indicative of its role in regulating drug response. We further showed that NFAT2 played a critical role in promoting cell proliferation and overcome carboplatin-induced DNA damage and cell cycle arrest. NFAT2 knockdown or inhibition of its nucleus translation via cyclosporine A largely restored the sensitivity to carboplatin in the resistant line by inducing DNA damage, blocking cell cycle progression and activating apoptotic cell death. We thus suggest that NFAT2 is a putative therapeutic target to overcome carboplatin resistance in lung cancers.

1. Introduction

Lung cancer is the leading cause of cancer death all over the world, and platinum-based chemotherapy (cisplatin, carboplatin) is the first-line treatment for patients with lung cancer (Ettinger et al., 2017; Gridelli et al., 2015; Siegel et al., 2018). The molecular mechanism of platinum-based chemo drugs involves crosslinking DNA and interfering with DNA replication in tumor cells, which in turn induces cell cycle arrest and activates programmed cell death if the DNA damage can't be repaired (Dasari and Tchounwou, 2014; Wang and Lippard, 2005). However, in clinical practice, intrinsic and acquired resistance to those agents remarkably reduces the therapeutic efficacy and has emerged as a serious impediment to lung cancer treatment. Over the past several decades, many efforts have been placed to understand the mechanism of platinum drug resistance and develop therapeutic strategies to restore drug sensitivity (Chabner and Roberts Jr., 2005; Stewart, 2007).

Nucleus factor of activated T cells, cytoplasmic 1 (NFAT2) is a transcription factors important in immune response. Recently, NFAT2 has been recognized as an oncogene implicated in many aspects of tumor biology (Quang et al., 2015; Robbs et al., 2008). In the cell, cytoplasmic NFAT2 is dephosphorylated by calcineurin and then translocates to nucleus to initiate the transcription of genes involved in cell proliferation, survival, metastasis and drug resistance, while inhibiting calcineurin activity with cyclosporine A (CsA) and tacrolimus (FK506) has shown antitumor potential by blocking NFAT2 nucleus translocation and consequent target gene expression (Quang et al., 2015). In nucleus, activated NFAT2 has been found to induce anchorage-independent growth in various cancer cells through initiating c-Myc and cyclin D transcription (Buchholz et al., 2006; Singh et al., 2010). In addition, constitutively activated NFAT2 inhibits cell differentiation and enhances colony formation in fibroblasts cells, which further lead to malignant transformation (Neal and Clipstone, 2003; Robbs et al., 2008). Moreover, NFAT2 also interacts with NF-κB to maintain lymphoma cell survival by overexpressing CD154 and B-lymphoma stimulator and facilitates the metastasis of mouse bone tumor cells via MMP-2 upregulation (Pham et al., 2005; Velupillai et al., 2010). Notably, NFAT2 has been involved in imatinib resistance in CML cells by promoting IL-4 expression and contributes phosphorsulindac resistance and poor prognosis in pancreatic cancer (Gregory et al., 2010; Kantarjian et al., 2002; Murray et al., 2014).

In lung cancer, NFAT2 was found to support cancer cell survival by overexpressing DNA damage-induced apoptosis suppressor (DDIAS) (Im et al., 2015), and mediate resistance to gefitinib and erlotinib by modulating IL-6 and IL-4 expression through STAT3 pathway and

Abbreviations: NFAT2, nucleus factor of activated T cells cytoplasmic 1; CsA, cyclosporine A; DDIAS, DNA damage-induced apoptosis suppressor; HR, hazard ratios; CI, confidence intervals

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supporting cancer cell survival under anti-cancer drug treatment (Harada et al., 2014; Kundumani-Sridharan et al., 2012; Lagunas and Clipstone, 2009; Pao and Chmielecki, 2010). Recent evidences revealed that through activating the transcription of DDIAS, NFAT2 can also contribute to the resistance to cisplatin, a platinum drug in lung cancer treatment (Im et al., 2016). Beyond cisplatin, carboplatin is another frequently used platinum agent for lung cancer patients, which carries out the anti-tumor functions in a similar way to cisplatin and is commonly preferred due to its less toxicity (Adams et al., 1989). However, up to now, little information is available regarding the association between NFAT2 and carboplatin. We are still far from completely understanding the underlying mechanism of carboplatin resistance in lung cancer treatment and developing therapeutic strategies to restore drug sensitivity. In the current study, we thus investigate the relationship between NFAT2 and carboplatin resistance in lung cancer, and explore the potential methods to overcome carboplatin resistance by manipulating NFAT2.

2. Material and methods

2.1. Carboplatin resistant lung cancer cell line

Carboplatin resistant lung cancer cell line was generated from H460 cells using the pulse method as previously described (Barr et al., 2013). Briefly, H460 cells were cultured in RPMI-1640 medium (HyClone) supplemented with 10% fetal bovine serum, and were treated 1 μM carboplatin (Sigma-Aldrich) for 72 h. Drug was then removed and cells were allowed to recover for another 72 h. The same process was repeated for 1 month, and 2, 4, 8, 16 μM carboplatin was used for month 2–5. The resistant cells were continued cultured in 16 μM carboplatin for 3 months before they were used.

2.2. MTT assay

5×10^3 lung cancer cells were seeded in 96-well plates and allow to adhere at 37 °C for 12 h. Cells were then treated with carboplatin at different concentrations (0–1000 μM) for 72 h. MTT reagent was then added into each well. After incubation at 37 °C for 3 h, cells were treated with DMSO and cell viability was determined by absorbance recorded at 590 nm. Experiment was performed with five replications.

2.2.1. Apoptosis assay

Lung cancer cells under different treatment conditions (siRNA and CsA treatment) were incubated with or without 1 μM carboplatin for 72 h. After trypsinization, cells were harvested and washed twice with ice cold phosphate-buffered saline (PBS). After double staining with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 15 min, cells were analyzed by flow cytometry. Experiment was performed with three replications.

2.2.2. Cell cycle analysis

Lung cancer cells under different treatment conditions (siRNA and CsA treatment) were harvested by trypsinization after incubation with or without 1 μM carboplatin for 72 h. After being washed twice with ice cold PBS, cells were fixed overnight with 70% ethanol at −20 °C. Fixed cells were then treated with RNase, stained with PI and analyzed by flow cytometry. Experiment was performed with three replications.

2.2.3. Subcellular fractionation

Lung cancer cells cultured in 10 cm plates were harvested at 80–90% confluence by scraping into 500 ul fractionation buffer (20 mM HEPES, pH 7.4, 10 mM KCl, 2 mM MgCl2, 1 mM DTT, 1 mM EDTA, 1 mM EGTA) and homogenized 10–15 times using 1 mL syringe with a 27-gauge needle. Cell lysate was separated into pellet and supernatant after being centrifuged at 700 × *g* for 10 min. The pellet was washed twice with fractionation buffer to get nucleus fraction. Cytoplasm was obtained by centrifuging supernatant at 10,000 g for 10 min to remove mitochondrial pellet.

2.2.4. Western blot

20 μg protein mixture extracted from lung cancer cell, nucleus and cytoplasm with RIPA buffer were separated by 10% SDS-PAGE and transferred onto methanol PVDF membrane. Expression level of actin, lamin B, NFAT2 and γ-H2AX was detected with corresponding antibodies and visualized by the chemiluminescence method with the ECL Plus Western Blotting Detection System (GE Healthcare).

2.2.5. NFAT2 knockdown

2×10^5 lung cancer cells were seeded in 6-well plates and allow to adhere at 37 °C for 20 h. siRNA oligos against NFAT2 (siNFAT2) and negative control (siNC) (Thermo Fisher Scientific) were delivered into lung cancer cells with lipofectamine 2000 (Invitrogen), followed by incubation at 37 °C for 72 h.

2.2.6. Cyclosporine A (CsA) treatment

H460/Car cells were incubated with CsA (Sigma-Aldrich) at a concentration of 0–15 μM for 48 h and were evaluated for viability, apoptosis, cell cycle distribution. Subcellular location of NFAT2 was determined after subcellular fractionation. The expression of NFAT2 targets were measured by RT-PCR.

2.2.7. Caspase activity

The enzymatic activity of caspase-2 and caspase-3 were assessed in lung cancer cells incubated with or without 1 μM carboplatin for 72 h using Caspase 2 and Caspase 3 Assay Kits (Abcam). Briefly, for Caspase 2 activity assay, substrate VDVAD-AFC is cleaved by caspase-2 to generate free AFC which could be detected by microtiter plate reader by reading absorbance at 505 nm. For Caspase-3, the assay is based on cleavage of the labeled substrate DEVD-pNA to generate free p-NA, which can be quantified using a microtiter plate reader reading absorbance at 400 nm. Experiment was performed with three replications. Experiment was performed with five replications.

2.2.8. RT-PCR

Total RNA was extracted from lung cancer cells treated under different conditions (siRNA and CsA) using Trizol reagent (Invitrogen) and was reverse transcribed into cDNA using the on-step Reverse Transcription kit. The cDNA was then amplified with SYBR Green PCR kit and loaded on CFX96 Real-time PCR Detection System (Bio-Rad). The primers used in the study include:

Cyclin D1:
5′-ATGTTCGTGGCCTCTAAGATGA-3′.
5′-CAGGTTCCACTTGAGCTTGTTC-3′.
IL-2:
5′-GAATCCCAAACTCACCAGGATGCTC-3′.
5′-TAGCACTTCCTCCAGAGGTTTGAGT-3′.
IL-4:
5′-ACATTGTCACTGCAAATCGACACC-3′.
5′-TGTCTGTTACGGTCAACTCGGTGC-3′.
GAPDH:
5′-CCTGCACCACCAACTGCTTA-3′.
5′-GGCCATCCACAGTCTTCTGAG-3′.

2.2.9. Data analysis

Survival curves were constructed with the Kaplan-Meier method and compared using log-rank test using the webtool Kaplan-Meier Plotter tool. It integrated transcriptome data of 720 lung adenocarcinomas from the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) and data source is described in (Gyorffy et al., 2013). All statistical analysis was performed using PRISM 6 (GraphPad, CA, USA).

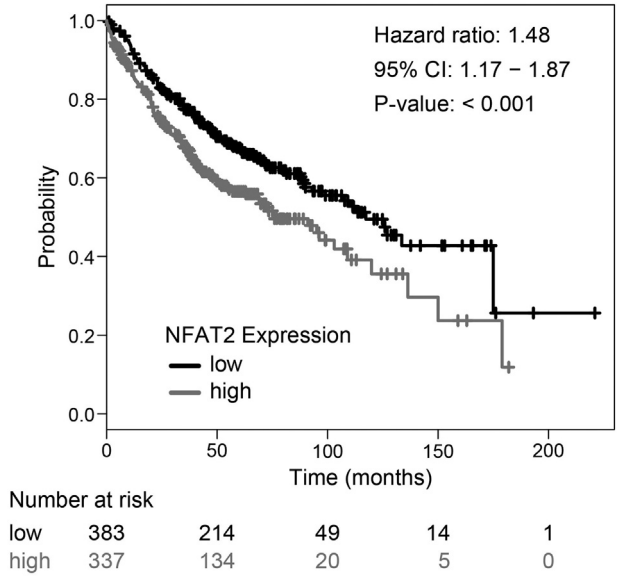

Fig. 1. The overall survival of lung cancer patients with high (grey curve) and low (Black curve) NFAT2 expression. Data analysis was performed using Kaplan-Meier Plotter tool. High or low group was stratified by median expression of NFAT2.

3. Results

3.1. NFAT2 overexpression was associated with poor prognosis of lung cancer

We first evaluated the association between NFAT2 expression and the overall survival of lung cancer patients using Kaplan-Meier Plotter tool (Gyorffy et al., 2013), which integrated transcriptome data of 720 lung adenocarcinomas from the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA). The intensities of the probes assigned to NFAT2 were used to estimate the relative NFAT2 abundance in each patient. Patients with NFAT2 expression above average were classified as high NFAT2 group while the others were in low NFAT2 group. The patients were followed for up to 17 years and the survival of patients in each group was evaluated by Kaplan-Meier method with log-rank test to determine statistical significance. Cox proportional-hazards model was used to estimate hazard ratios (HR) and 95% confidence intervals (CI). As shown in Fig. 1, patients with high NFAT2 levels were observed with reduced overall survival compared to those with low NFAT2 levels (HR: 1.48, 95% CI: 1.17–1.87, P-value: < 0.001), suggesting the association of NFAT2 with poor prognosis of lung cancer.

3.2. NFAT2 was associated with carboplatin resistance in lung cancer

Poor prognosis is usually correlated with treatment failure and cancer relapse. Since carboplatin is one of the first-line treatments for lung cancer patients, we then investigated the association of NFAT2 with carboplatin response in lung cancer cells. Carboplatin resistance lung cancer cell line H460/Car was developed from H460 following continuous exposure to carboplatin (see schematic in Fig. 2A). As compared with the parent H460 cell line, H460/Car cells demonstrated significantly increased proliferation rate (Fig. 2B) and potently reduced sensitivity to carboplatin treatment (Fig. 2C, IC_{50} of H460/Car: 29.38 μM vs. IC_{50} of H460 cells: 1.05 μM). Of note, treatment with carboplatin resulted in enhanced cell cycle arrest at G2/M phases (Fig. 2D–E) and stronger signals of DNA damage marker γ-H2AX (Fig. 2F) in H460 cells versus H460/Car cells. Consistently, we observed significantly lower number of apoptotic cells in drug-treated H460/car versus H460 cells (Fig. 2G–H), and the activities of apoptosis regulators caspase-3 and caspase-2 were likewise compromised (Fig. 2I), all supporting reduced apoptotic cell death. All these results supported that we have successfully established a carboplatin resistant H460/Car cell line.

We then assess NFAT2 expression and activity in carboplatin resistant cells. As shown in Fig. 3A, the overall NFAT2 expression was higher in carboplatin resistant H460/Car cells than H460 cells. Of note, in H460/Car cells, NFAT2 mainly located in nucleus fraction rather than in cytoplasm, while in H460 cells, more NFAT2 was detected in cytoplasm. Given that NFAT2 is activated by dephosphorylation and nucleus translocation, this result suggested enhanced NFAT2 activity in carboplatin resistant lung cancer cells. Activated NFAT2 was known to induced the transcription of genes involved in cell cycle. In our study, we found that cell cycle gene cyclin D1 was highly expressed in H460/Car cells (Fig. 3B), which may confer resistance to carboplatin-mediated apoptosis through enhancing DNA damage repair and promoting cell cycle progression (Jirawatnotai et al., 2012; Shah and Schwartz, 2001). We also found that cytokine IL-2 and IL-4 were also overexpressed in H460/Car cells (Fig. 3B), which may further support the survival of cancer cells under carboplatin treatment (Jones et al., 2016).

3.3. NFAT2 silencing resensitized lung cancer cells to carboplatin treatment

We then asked if reducing NFAT2 expression could reverse the carboplatin resistant phenotype in H460/Car. H460/Car cells were transfected with NFAT2 or control siRNAs for 24 h and then treated with 1 μM carboplatin for 72 h. Compared to control knockdown, H460/Car cells with NFAT2 knockdown exhibited increased level of γ-H2AX in response to carboplatin treatment (Fig. 4A), and the expression levels of NFAT2 targets cyclin D1, IL-2 and IL-4 were significantly reduced (Fig. 4B). Consistently, enhanced G2/M arrest (Fig. 4C–D) and elevated cell death (Fig. 4E–F) were observed in NFAT2 knockdown cells versus control knockdown upon drug treatment (Fig. 4C–E), and the activity of apoptosis regulators caspase-3 and caspase-2 was enhanced (Fig. 4G). As revealed by cell survival analysis, IC_{50} carboplatin concentration was largely reduced from 31.10 μM in control cells to 1.46 μM in siNFAT2 treated cells (Fig. 4H). These results revealed restoration of carboplatin sensitivity in lung cancer cells following NFAT2 silencing, confirming the function of NFAT2 in carboplatin drug resistance.

3.4. Inhibiting NFAT2 unclear translocation restored carboplatin sensitivity

NFAT2 carries out its function as a transcriptional factor after nucleus translocation and more nucleus NFAT2 was observe in carboplatin resistant H460/Car cells (Fig. 3A). We therefore hypothesized that inhibiting NFAT2 nucleus translocation may also help to resensitize lung cancer cells to carboplatin. To this end, H460/Car cells were treated with increased concentrations of cyclosporine A (CsA), which blocked NFAT2 dephosphorylation in cytoplasm and resulted in decreased NFAT2 accumulation in nucleus (Fig. 5A). Accordingly, the expression of cyclin D1, IL-2 and IL-4 in H460/Car cells was reduced in relation to CsA concentration (Fig. 5B). Moreover, CsA also increased the sensitivity to carboplatin in a dose dependent manner. As evidenced by increased γ-H2AX levels, CsA treatment lead to the dose-dependent accumulation of DNA damage on carboplatin treatment (Fig. 5A), which in turn resulted in cell cycle arrest at G2/M phases (Fig. 5C–D) and finally activated apoptotic caspase cascade (Fig. 5E–G). A dose-dependent reduction of IC_{50} carboplatin concentration was observed in CsA-treated H460/Car cells (Fig. 5H). We therefore concluded that inhibiting NFAT2 activity can also resensitize lung cancer cells to carboplatin.

4. Discussion

NFAT is a ubiquitous transcription factor and its dysregulation may cause abnormal expression of various target genes involved in cancer

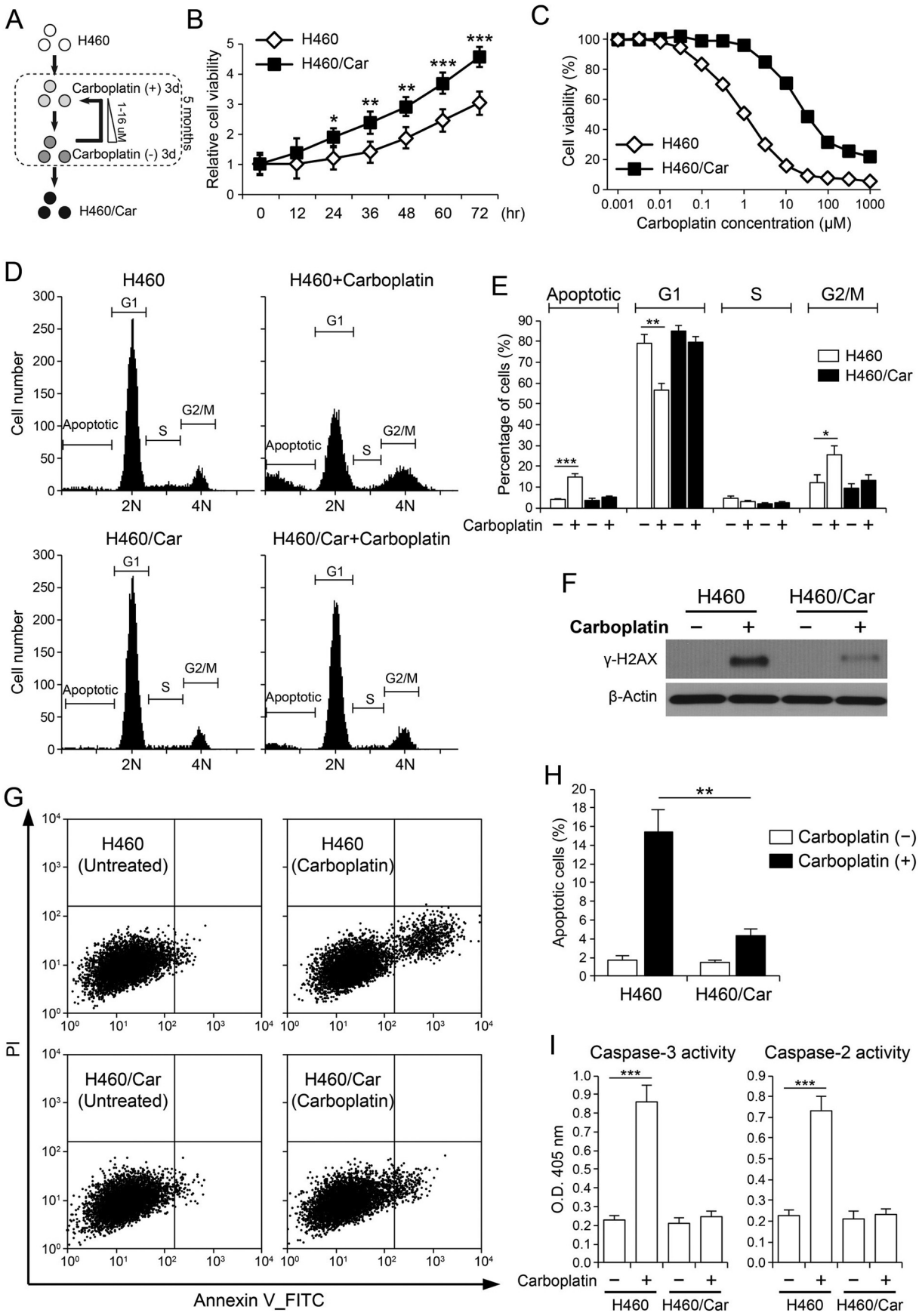

Fig. 2. H460/Car cells demonstrated impaired carboplatin sensitivity compared to parent H460 cells. (A) Schematic of establishment of the carboplatin-resistant cell line H460/Car. (B) Proliferation rate of H460 and H460/Car cells. (C) The viability of H460 and H460/Car cells on carboplatin treatment. (D, E) Cell cycle distribution of H460 and H460/Car cells with and without carboplatin treatment. (F) The expression level of DNA damage marker, γ-H2AX. (G, H) Apoptotic analysis of H460 and H460/Car cells with and without carboplatin treatment. (I) Caspase-3 and Caspase-2 activity in H460 and H460/Car cells treated with and without

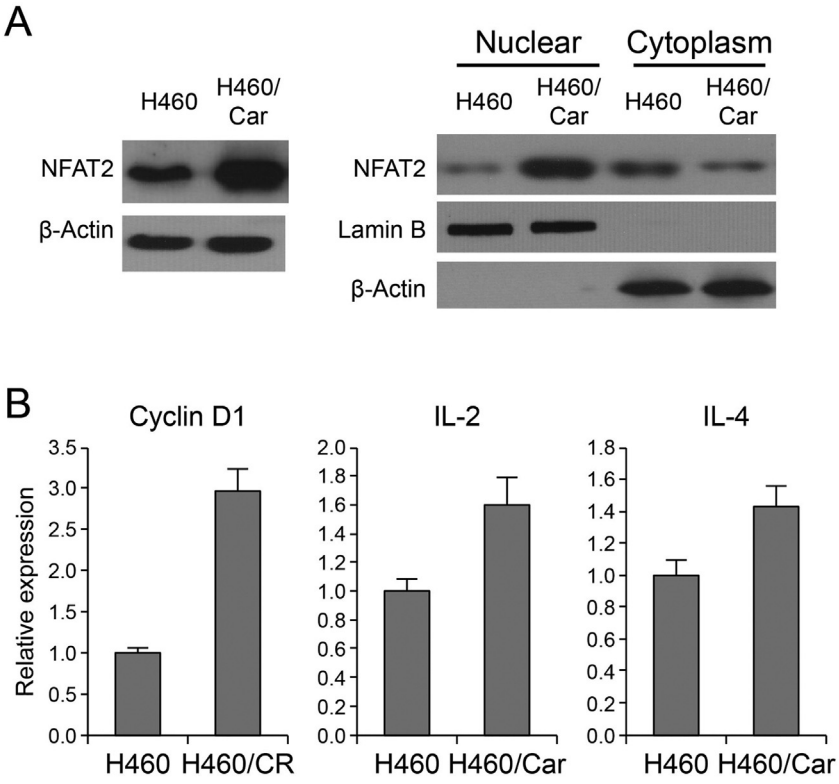

Fig. 3. NFAT2 was overexpressed and activated in carboplatin resistant lung cancer cells. (A) The expression and subcellular location if NFAT2 in H460 and H460/Car cells. (B) The expression of NFAT2 target genes in H460 and H460/Car cells.

progression. In the current study, we explored the association of transcription factor NFAT2 with carboplatin resistance in lung cancer. We found that carboplatin resistance was associated with increased NFAT2 expression and enhanced NFAT2 nucleus translocation. NFAT2 is known to regulate the transcription of genes involved in various cellular processes, including inflammation, proliferation, angiogenesis and metastasis. We found that activated NFAT2 may induce the overexpression of cell cycle genes (e.g. cyclin D1) in carboplatin resistant lung cancer cells, which may confer drug resistance by accelerating cell proliferation and relieving carboplatin-induced cell cycle arrest (Jirawatnotai et al., 2012; Shah and Schwartz, 2001). In addition, enhanced expression of cytokines (e.g. IL-2 and IL-4) may further constitute inflammatory tumor microenvironment to support cancer progress and enhance the viability of lung cancer cells under carboplatin treatment (Jones et al., 2016). It has been shown that constitutively activated NFAT2 not only induces cell transformation but also promotes colony formation in fibroblasts (Neal and Clipstone, 2003). It has also been shown that NFAT2 may induce c-Myc transcription, leading to cyclin D upregulation and anchorage-independent growth (Buchholz et al., 2006). All these results suggest a contributory role of NFAT2 in impaired response to carboplatin in lung cancer patients. Moreover, we also provided evidences that silencing NFAT2 expression by siRNA and inhibiting NFAT2 nucleus translocation by cyclosporine A can effectively restore carboplatin-mediated apoptosis in lung cancer cells and could become novel therapeutic strategies to overcome carboplatin resistance in clinical practice.

However, as demonstrated by extensive evidence, the development of resistance to carboplatin and other platinum agents in cancer cells is a complex process involving profound changes at various biological levels. For example, in addition to NFAT2-related cell cycle and inflammation alterations observed in our study, activated glutathione biosynthesis has also been associated with carboplatin resistance through acting on detoxification and cell protection, while inhibiting the expression of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme in glutathione production, has shown potency in restoring carboplatin sensitivity (Caffrey and Frenkel, 2013; Lopes-Coelho et al., 2016). Similarly, many other detoxification systems, such as metallothioneins, antioxidants and stress response chaperones, can also be activated by platinum agents, which in turn protect cancer cells from drug-mediated apoptosis and lead to resistant phenotype (Knipp, 2009; Shen et al., 2012). Moreover, membrane transporting system has also been associated with platinum drug resistance by inhibiting drug uptaking and enhancing the efflux process (Beretta et al., 2010; Howell et al., 2010; Shen et al., 2010). And many other transcription factors have also been implicated in platinum response by regulating the expression of genes involved in various biological functions, such as Y-box binding protein-1, CCAAT-binding transcription factor 2, activating transcription factor 4, zinc-finger factor 143, the nucleus transcription factor-κB, the microphthalmia-associated transcription factor, the forkhead transcription factor O, and mitochondrial transcription factor A (Lee et al., 2009; Lei and Quelle, 2009; Torigoe et al., 2005). Notably, aberrant DNA hypermethylation and histone acetylation have also been reported to induce platinum resistant in cancer cells by affecting gene expression, while inhibiting DNA methylation and histone acetylation can reverse drug resistance (Chang et al., 2010; Koul et al., 2004; Steele et al., 2009; Wermann et al., 2010), suggesting that platinum resistance may also be regulated at epigenetic level. More recently, numerous studies have revealed a possible role of microRNA in tumor growth and response to chemotherapy by targeting diverse genes implicated in apoptosis, cell motility and invasiveness, implying the involvement of noncoding RNA in the development of chemoresistance (Hamano et al., 2011; Imanaka et al., 2011; Yang et al., 2008; Zhu et al., 2012). All these evidences suggested the complexity of developing platinum resistance in cancer cells, and we are still far from complete understanding the molecular mechanism of platinum resistance in lung cancer.

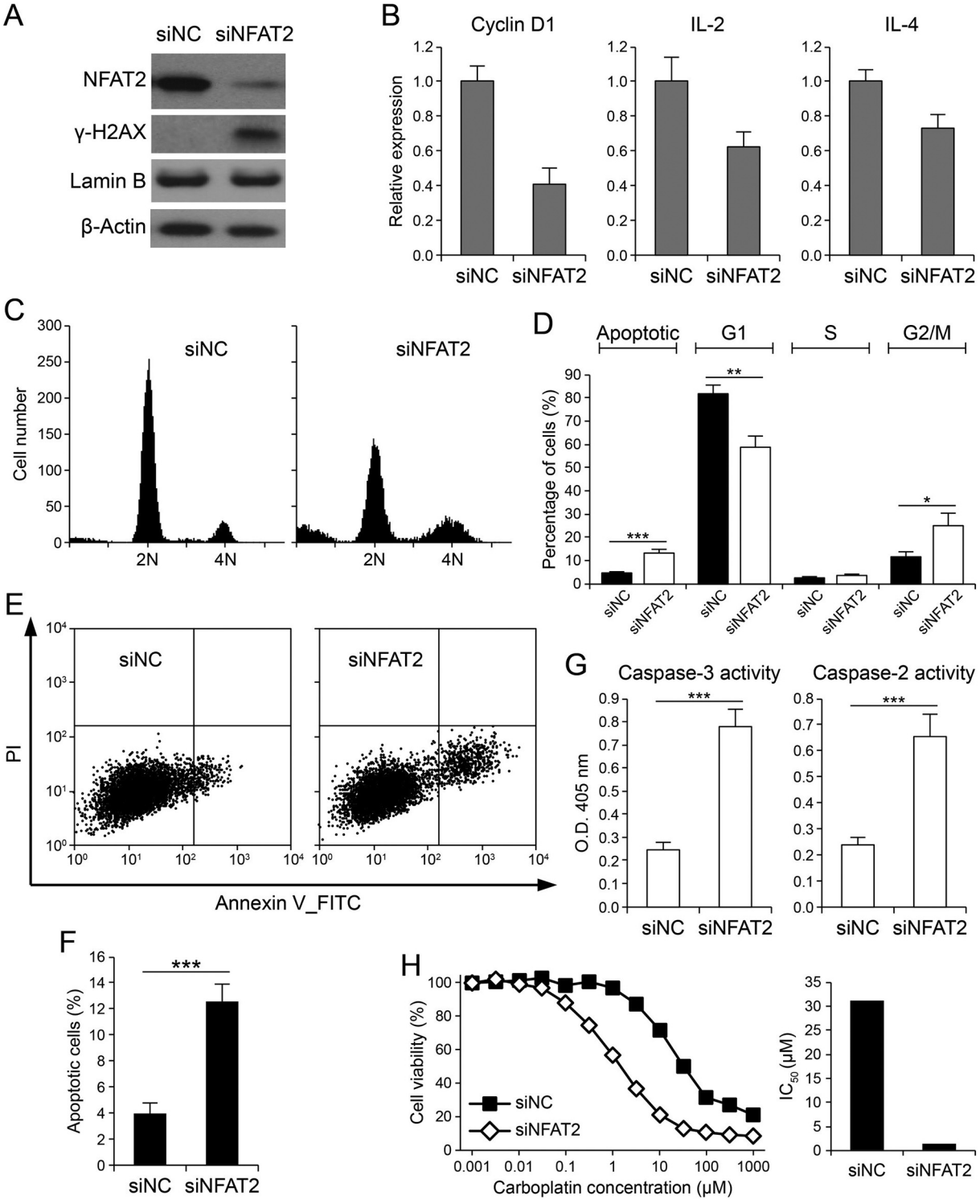

Fig. 4. NFAT2 silencing resensitized lung cancer cells to carboplatin treatment. (A) NFAT2 expression in H460/Car cells was knocked down by siRNA. (B) The expression of NFAT2 target genes in H460/Car cells was reduced following NFAT2 silencing. (C, D) NFAT2 silencing restored carboplatin-induced cell cycle arrest in H460/Car cells. (E, F) NFAT2 silencing restored carboplatin-induced apoptotic cell death in H460/Car cells. (G) NFAT2 silencing increased Caspase-3 and Caspase-2 activity in H460/Car cells on carboplatin treatment. (H) NFAT2 silencing restored carboplatin sensitivity in H460/Car cells.

5. Conclusions

Our study provided evidence that NFAT2 is associated with carboplatin resistance in lung cancer and could become a promising therapeutic target. However, more efforts are still needed to expand our knowledge on platinum-based chemotherapy and develop novel strategy to improve treatment efficacy.

Declaration of Competing Interest

None.

Acknowledgements

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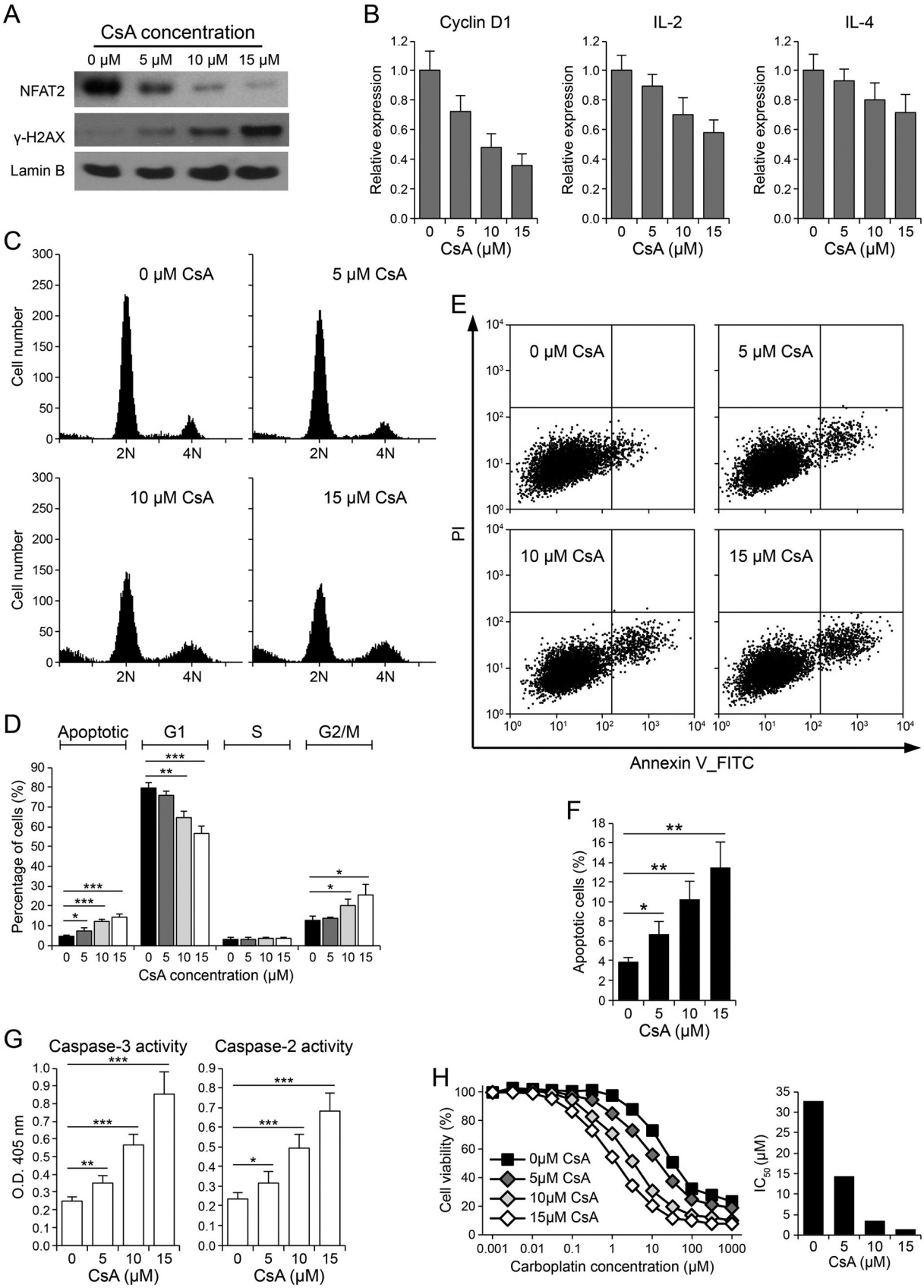

Fig. 5. Inhibiting NFAT2 unclear translocation restored carboplatin sensitivity. (A) CsA treatment inhibited NFAT2 accumulation in nucleus in H460/Car cells. (B) The expression of NFAT2 target genes in H460/Car cells was reduced following CsA treatment. (C, D) CsA treatment restored carboplatin-induced cell cycle arrest in H460/Car cells. (E, F) CsA treatment restored carboplatin-induced apoptotic cell death in H460/Car cells. (G) CsA treatment increased Caspase-3 and Caspase-2 activity in H460/Car cells on carboplatin treatment. (H) CsA treatment restored carboplatin sensitivity in H460/Car cells.

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