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Original Article

The effect of Baicalein against lung cancer depends on GADD45a down-regulation

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Abstract: Lung cancer is one of the most universal cancers in the world. Although baicalein has been used as an anticancer reagent in lung cancer cell, the precise molecular mechanism still remains unclear. In present study, we provide the mechanistic link between baicalein and its anti-lung cancer activity. Primarily, baicalein inhibits the cell proliferation and induces cell cycle arrest, which is accompanied by the down-regulated expression of CDK4, Ki67 and PCNA. Using RNA-seq, we find that baicalein regulates the expression of nineteen genes in A549 cell, in which GADD45a (growth arrest and DNA-damage-inducible protein 45 alpha) presents a relatively lower expression. The GADD45a expression reduced by baicalein is repeatedly identified by western blotting and RT-PCR analysis, revealing that GADD45a may be a central downstream target of baicalein. Consistently, we demonstrate subsequently that over expression of GADD45a reverses the anticancer effect of baicalein on A549 cell, revealing the negative relationship between expression of GADD45a and anticancer action of baicalein. Collectively, we suggest that cell cycle arrest and lower cell proliferation induced by baicalein depend largely on down-regulated expression of GADD45a, and GADD45a is a potential biomarker for modulation or therapeutic intervention of lung cancer.

Keywords: Lung cancer, baicalein, RNA-seq, GADD45a

Introduction

Despite our efforts to halt the incidence of lung cancer, it continues to become the leading cause of cancer-related death in men and women [1]. On an annual basis, lung cancer leads to more than 1,000,000 deaths worldwide [1, 2]. Based on the classification by WHO, lung cancer has two broad histological subtypes: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC), the latter can be further classified into adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma [3]. So far, surgery and drug-based chemotherapy is still the standard treatment for people with advanced lung cancer [4, 5]. Although these therapies extended the overall survival, the treatment of patients with lung cancer remains a therapeutic challenge because of its unresponsiveness to conventional chemotherapeutic and biological reagents, which has been attributed to development of resistance to apoptosis [6, 7]. Therefore, a becoming solution is to identify new therapeutic agents and strategies that improve the outcomes of lung cancer patients.

Earlier studies has revealed that scutellaria baicalensis (common name: Huang-Qin in China), a herbal medicine, is a broad spectrum, anticancer agent that induces apoptosis through enhanced cytotoxicity [8-10]. Accumulating reports indicated that three clinically important bioactive components, baicalin, baicalein and wogonin, may account for the cytotoxicity of S. baicalensis in various cancer cell lines in vitro [11-13]. Although the potential anticancer activity of baicalein has been speculated in lung cancer [13], molecular mechanism by which baicalein acts is poorly understood. In other human cancer cell line, the anticancer mechanism of baicalein may be linked to the potent cell cycle arresting and apoptotic properties [14], which provides the clue for illuminating the anticancer role of baicalein in lung cancer cell.

Generally, cellular phenotypes possess differential gene expression profiles. Comprehensive

gene expression profiling analysis boost understanding of how gene expression transform phenotypic representation, and facilitates a better explanation of the molecular mechanism for antineoplastic modulated by complicated genetic and biochemical networks [15, 16]. Although microarray is the most predominant hybridization-based approach to measure gene expression due to their capability to explore thousands of transcripts simultaneously [17], it is subject to several biases and limitations including hybridization kinetics, probe selection, background hybridization and cross platform comparability [18]. Now, RNA-seq, a main quantitative transcriptome profiling platform, has been created to overcome these shortcomings. Given the high depth of read coverage, RNA-seq not only causes a more accurate survey for levels of transcripts and their isoforms, but also has an ability to investigate transcript structures in context of transcription start sites, alternative splicing patterns and other post-transcriptional modifications [15].

In the present study, we primarily evaluated the effect of baicalein on proliferation and cell cycle distribution of human NSCLC A549 cell line. Then, using RNA-seq, we screened out the GADD45a which regulated by administration of baicalein. These results elucidated that exogenous baicalein significantly down-regulated the expression of GADD45a, which ultimately inhibits the proliferation and promotes the cell cycle arrest of A549 cell, revealing the importance of GADD45a in anticancer role of baicalein.

Materials and methods

Cell culture and treatment

Human NSCLC A549 cell line was maintained in a humidified 5% CO_2 incubator at 37°C in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% (V/V) fetal bovine serum (FBS) (Hyclone), 100 U/ml penicillin-streptomycin. Cells grown in cell culture dish were allowed approximately to reach 85% confluence. Monolayer cells were harvested using trypsin EDTA (Hyclone) and passaged.

Baicalein was prepared in DMSO in 25 μg/mL stock solution and kept at 4°C. After diluting with DMSO, the cells were treated with 10, 100 and 1000 nM of baicalein for 1, 3, 5 and 7 day. In addition, A549 cells were respectively transfected by pcDNA3 and pcDNA3-GADD45a plasmid, and then treated with baicalein (100 nM). All treated cells were used for subsequent study.

Cell proliferation assay

Cell proliferation was determined with a Cell-Counting CCK-8 assay kit (Dojindo) according to manufacturer protocols. A549 cells were cultured into 96-well plates at the density of 6×10^5 cells/well and cultured for 24 h. After incubating with indicated conditions, 5 μl CCK-8 reagent was added to each well and incubated at 37°C for 1 h. The cells were harvested and then were evaluated by measurement of absorbance at 450 nm.

Flow cytometric analysis

After digesting, A549 cells were directly plated at a density of 6×10^5 cells/well in 96-well plates. Prior to incubation, the cells were cultured overnight. Cell cycle analysis was performed by using Cell Cycle Kit according to the manufacturer introductions. In particular, A549 cells were respectively treated with DMSO or different concentrations of baicalein. The cells were harvested by trypsin and washed twice with PBS. Then the results were analyzed by Muticycle for windows software.

Western blotting

For immunodetection, A549 cells were lysed directly in RIPA lysis buffer and boiled for 10 min. After the removing of the insoluble fraction by centrifuge, 50 μg of total protein extracts was resolved on 10% SDS-PAGE, which was then transferred to nitrocellulose membranes for Western blotting. The membranes were first stained with Ponceau S to confirm the transfer efficacy. After blocking with 5% non-fat dry milk dissolved in Tris-buffered saline (TBS), containing 0.05% Tween-20 (TBST), for 1 hour at 37°C, membranes were incubated with the CDK4, Ki67, PCNA, GAPDH, GADD45a and GADD45b antibodies at a dilution of 1:1000, followed by goat anti-rabbit secondary antibody conjugated with horse radish peroxidase at 1:2,000 dilutions. These antibodies were all purchased from Santa Cruz or Abcam. Positive band intensities were detected by using a gel documentation system (LAS-3000 Fujifilm).

RNA-seq library preparation and sequencing

RNA-seq protocol was performed as previously described [15], with minor modifications. Briefly, total RNA was isolated from DMSO- and baicalein-treated A549 cells using RNeasy Mini Kit (Qiagen, Valencia, CA). After examining the RNA quality and integrity, poly-T oligo-attached magnetic beads were used for mRNA cleanup from 2 mg total RNA. Purified mRNA was fragmented and then primed with random hexamers. First-strand cDNA was reverse transcribed from cleaved mRNA fragments using reverse transcriptase and random primers. Right before double-strand cDNA synthesis, second-strand cDNA was synthesized to replace the mRNA template. End repair, addition of a single A base, adaptor ligation, cDNA template purification and enrichment of the purified cDNA templates using PCR were then carried out. Following manufacturer's indications, constructed libraries were 100 bp paired-end sequenced by an IlluminaGAIIx sequencer on two lanes of the flow cell.

Quantitative RT-PCR assay

For quantitative RT-PCR assay, RNA was isolated from A549 cells using the TRIzol reagent according to the manufacturer's instructions. 2 μg of total RNA were provided to generate the first-strand cDNAs by using commercially available kits (Applied Biosystems). All subsequent PCR reactions were carried out using the 7 Universal PCR Master Mix (Applied Biosystems). Primers used for amplification were as follows: 5'-GACGAATCCACATTCATCTC-3' (sense) and 5'-TTGATCCATGTAGCGACTT-3' (antisense) for GADD45a; 5'-ATTGACGAGGAGGAGGAG-3' (sense) and 5'-GTTGTCACAGCAGAAGGA-3' (antisense) for GADD45b. Thermal cycling and fluorescence detection of mRNA were analyzed by 7500 real-time PCR System (Applied Biosystems). To normalize mRNA concentrations, mRNA levels of β-actin gene were identified in parallel for each sample, and relative mRNA level of DNMT3a was adjusted by standardization based on the β-actin transcriptional levels. Samples for each experimental condition were run in triplicate.

Bioinformatic analysis

Transformations and manipulations of RNA-seq data were done utilizing Excel. Hierarchical clustering (HCL) was performed with MeV 4.7.4. Multivariate statistical analysis included principal component analysis (PCA) and multi-dimensional scaling (MDS). PCA was performed in R using the prompt function, and used to discriminate sample patterns and minimize the influence of inter-individual variation. We used multi-dimensional scaling to define a two-dimensional representation of DMSO- and baicalein-treated A549 cell as calculated by 1-*r*, where *r* is the Person correlation coefficient in comparison of the relative normalized expression of all analyzed genes between DMSO- and baicalein-treated A549 cells.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) to assess the treatment effects by using DPS 3.2 software. The Student t test was used to determine the statistically significant differences in numbers with two significant levels (0.05 and 0.01). The results are shown as mean ± standard deviation (SD).

Results

Baicalein inhibits the proliferation of A549 cell

Previous studies demonstrated that baicalein suppresses the cell growth of cell lines of hepatocellular and breast carcinoma, and of lung carcinoma CH27 cell [19-21]. To further understand the anticancer effect of baicalein in another cell line of lung cancer, A549 cell was used to examine. Primarily, cell proliferation was measured by applying the CCK-8 assay, as shown in **Figure 1A**. The cell proliferation of A549 cell was inhibited significantly by treating with 10 nM baicaleinin a time-dependent manner when compared with DMSO treated group ($P < 0.05$), while this suppressing effect was not further enhanced with an increasing concentrations of baicalein (100 nM and 1 μM). Then, we explored the cell cycle distribution of baicalein-treated A549 cell using flow cytometry assay. As exhibited in **Figure 1B**, low concentration of exogenous baicalein induced a delay in the G1 transition ($P < 0.05$) and this delay represented a dosage-dependent manner. Meanwhile, the G1 enrichment was accompanied by a promotion in S phase cells ($P < 0.05$, **Figure 1B**). Finally, three vital proliferation-related proteins, cyclin-dependent kinase 4 (CDK4), Ki67 and proliferating cell nuclear antigen (PCNA), were selected to western blot analysis. We detected that the low concentra-

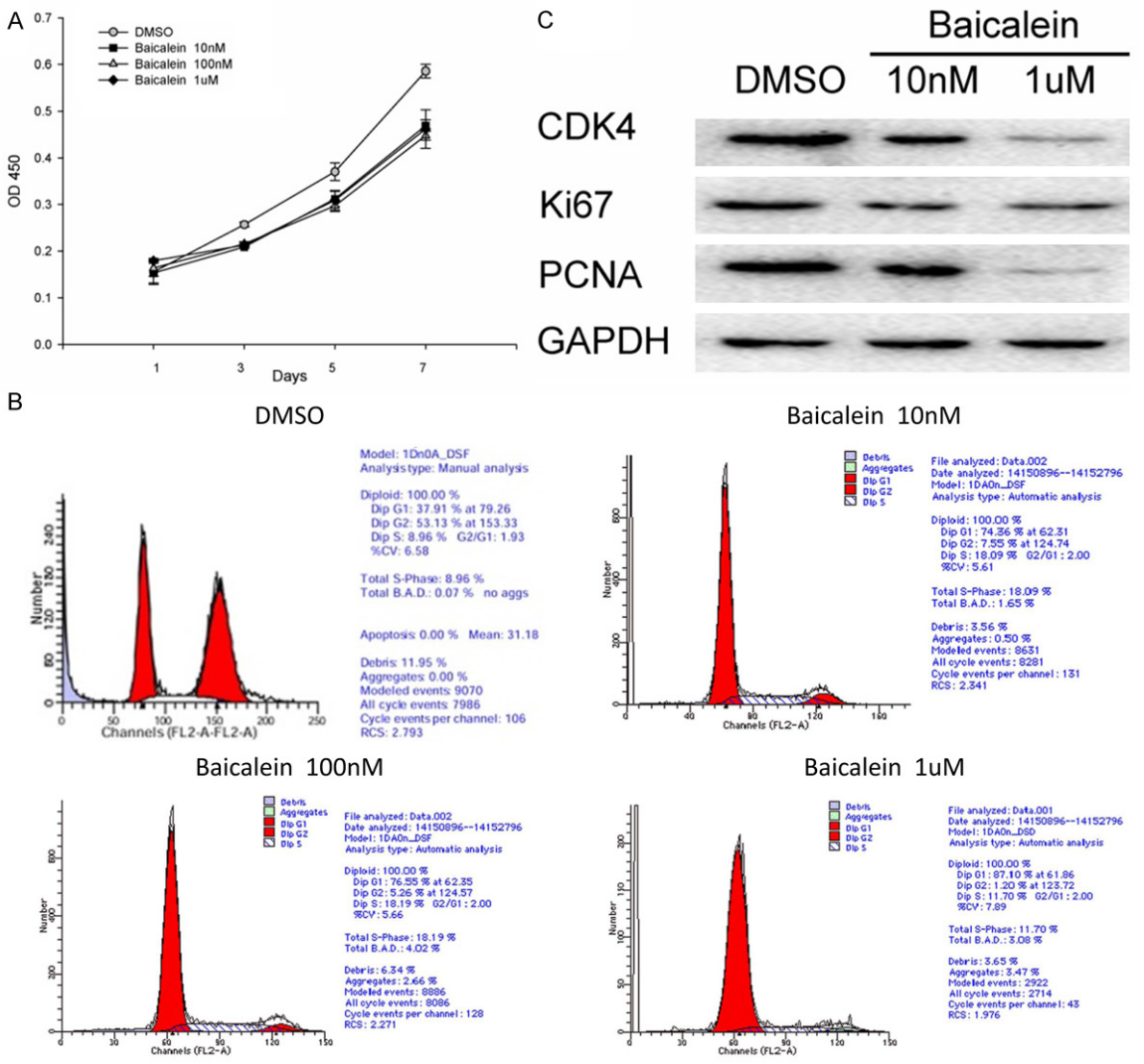

Figure 1. Baicalein inhibits A549 cell proliferation. A. CCK-8 assay of A549 cell in absence or presence of baicalein at indicated time. B. Effect of increasing concentrations of baicalein on cell cycle distribution. FACS histograms showed a number of cells per channel (vertical axis) vs. DNA content (horizontal axis). C. Western blot analysis of CDK4, Ki67 and PCNA expressions in A549 cells treated with DMSO, and 10 nM and 1 μM baicalein. $*P < 0.05$, $**P < 0.01$.

tion of baicalein initiates the down-regulated expressions of CDK4, Ki67 and PCNA, and high concentration of baicalein further inhibits the expressions of CDK4, Ki67 and PCNA (**Figure 1C**). Collectively, these data suggest that baicalein is an efficient anticancer agent that inhibits A549 cell proliferation through blocking the cell cycle.

Design of RNA-seq study in baicalein-treated A549 cells

To characterize the extent of expression changeability on a genomic scale and decipher its functional meaning, we would like to use RNA-seq to profile a temporal snapshot of the A549 cell response to baicalein. Upon the purpose of experiment, three experimental conditions, sequencing platforms, read lengths and sequencing types, which related to efficiency of RNA-seq analysis, should be properly determined. Based on previous studies, 100 bp paired-end sequencing was analyzed in preponderant IlluminaGAIIx platform by employing the constructed illumina RNA-seq library [15, 18, 22, 23]. A schematic diagram for the design and goals of our study is presented in **Figure 2**.

Transcriptomics data acquisition

Total numbers of RNA-seq reads (result format: FASTQ) acquired from DMSO- and baicalein-

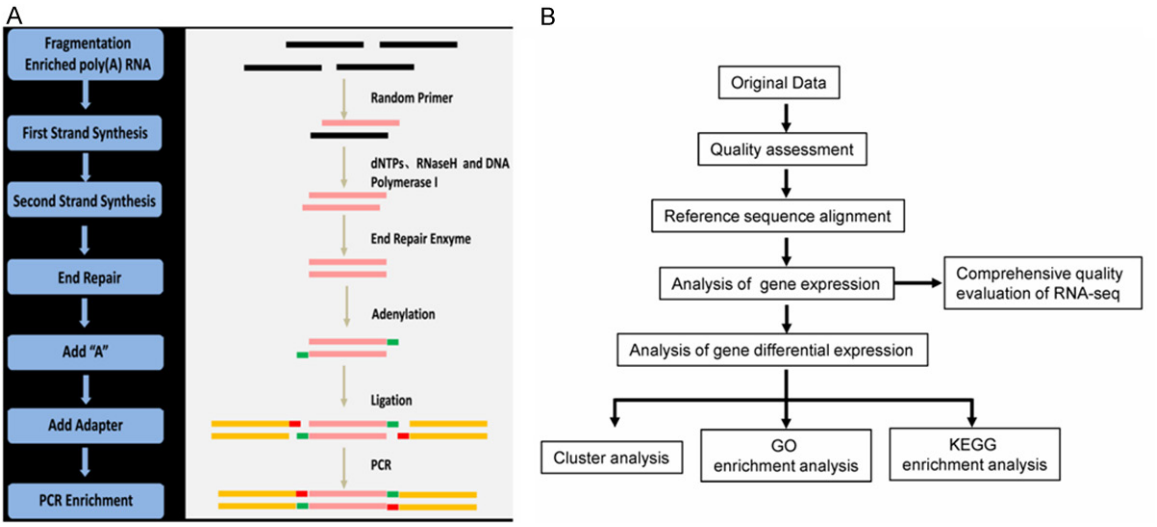

Figure 2. Schematic diagram for design and goals of our study.

Table 1. RNA-seq data of DMSO- and baicalein-treated A549 cells

Sample	Treatment	Raw Data Read	Base	Valid Data Read	Base	Valid% Read	Q30%	GC%
WGC028322	DMSO	2777469	2.64G	2728030	2.60G	98.22	90.93	50
WGC028325	Baicalein	2804384	2.67G	2758111	2.63G	98.35	90.67	50

treated A549 cells, were 2777469 and 2804384, respectively (**Table 1**). We aligned the sequence reads to a human genome reference (hg19) using TopHat version 1.2.0. Splice junctions were extracted from RefSeq alignment (downloaded from the University of California, Santa Cruz (UCSC) Genome Browser). In total, at least one ends of 2728030 and 2758111 reads for the DMSO- and baicalein-treated A549 cells, respectively, were successfully mapped against hg19. Next the resulting read aglinments (file format: BAM) were assembled through Cufflink version 1.0.3, and it created a novel transcript using Cufflinks reference annotation-based transcript (RABT) algorithm. The transcripts combined by Cuff compare were used to calculate relative abundances of each transcript through Cuffdiff. Gene expression levels were determined by measuring the sum off fragments per kilobase of exon model per million mapped reads (FPKM) values of its exons.

Acquisition of baicalein-regulated genes

To further explore a differential transcriptome identifying the baicalein-treated A549 cell from the DMSO-treated A549 cell, volcano plot analysis was used to determine differential genes. The volcano plot is an effective and easy-to-interpret graph that summarizes both *t*-test and fold-change criteria. After calculation, total genes were plotted by the negative $\log_{10}$-transformed *p*-values from the gene-specific *t* test against the $\log_2$ fold change (**Figure 3A**). Based on the given criteria of *p* value and fold-change, we found that baicalein regulates the expression of nineteen genes. Out of these genes, ten were increased while others were decreased in baicalein-treated cell (**Figure 3B** and **3C**). For above genes, generally, abundance was differentially increased in the cells that avail tumorigenesis (or vice versa). Given that, we primarily focus on the genes which can be reduced by baicalein treatment. Two of the nine down-regulated genes, *gadd45a* and *gadd45b*, presented the lowest expressions, indicating that *gadd45a* and *gadd45b* may be two central downstream targets of baicalein.

Baicalein reduces the expression of GADD45a

Next, we further confirmed the GADD45a and GADD45b expressions in baicalein-treated A549 cells from protein and mRNA levels. In protein level, untreated A549 cells highly

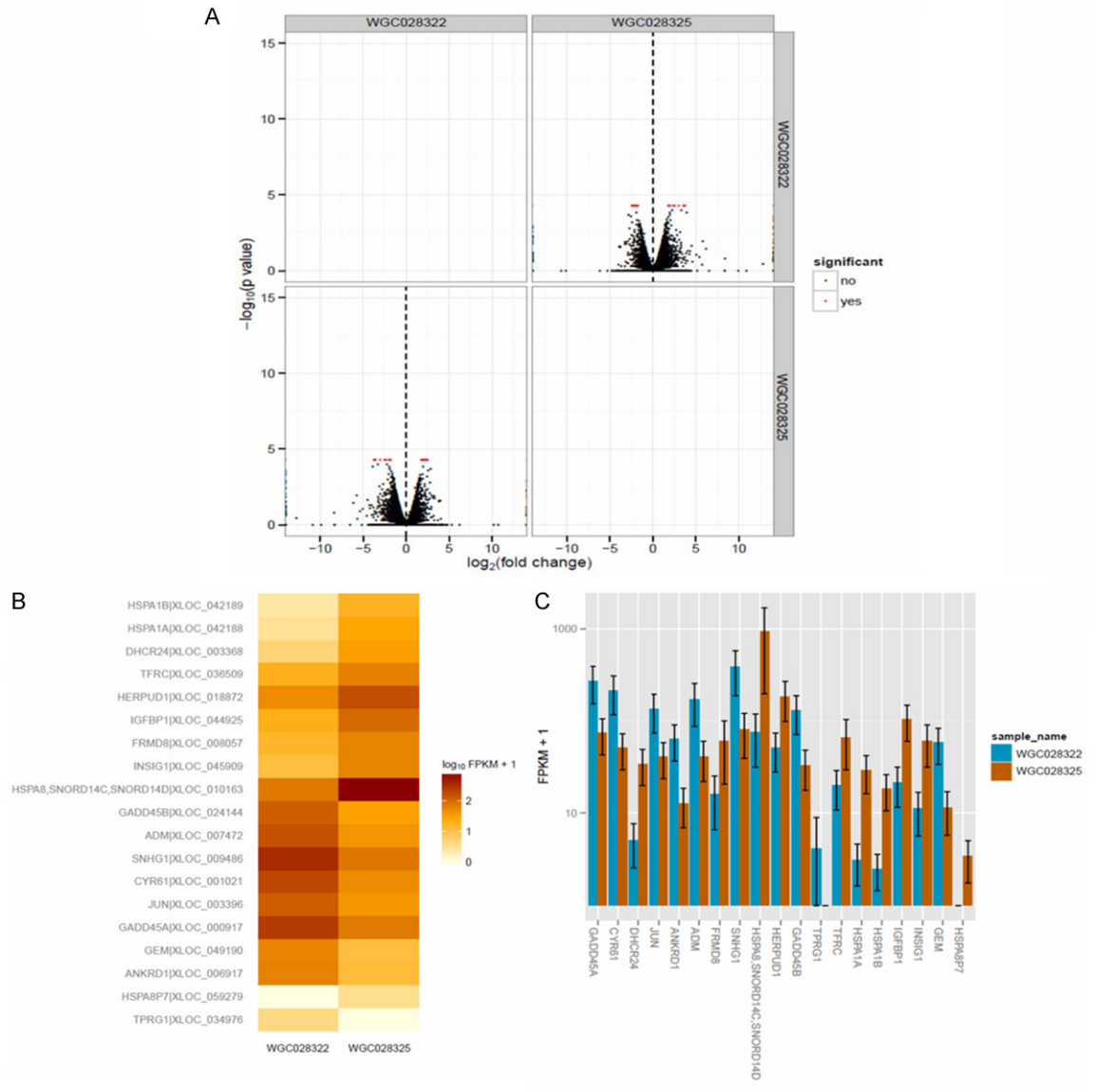

Figure 3. Screening of baicalein-regulated genes. A. Volcano plot analysis of differential genes from DMSO- and baicalein-treated A549 cell. B. HCL analysis of differential genes. C. Histogram of differential genes.

expressed GADD45a and GADD45b, while the expression of GADD45a markedly reduced, and the expression of GADD45b had no difference in A549 cells treated with three concentration of baicalein (**Figure 4A**). This finding suggested that baicalein have an ability to inhibit the GADD45a expression in A549 cell and this suppressing effect represented a dosage-dependent manner. We also examine the GADD45a and GADD45b expressions in A549 cell from transcriptional level. As shown in **Figure 4B** and **4C**, the expression of GADD45a was significantly inhibited in A549 cell treated with three increasing dose of baicalein in contrast to the untreated A549 cells ($P < 0.05$) although the mRNA level of GADD45b remains unaffected.

Over expression of GADD45a reverses the effect of baicalein on A549 cell

In a follow-up experiment, we investigate whether down-regulated GADD45a contributes to baicalein-induced inhibition of proliferation in lung cancer cell. To this end, we primarily established GADD45a recombinant plasmid and then constructed vector was transferred into A549 cells. The GADD45a expression was

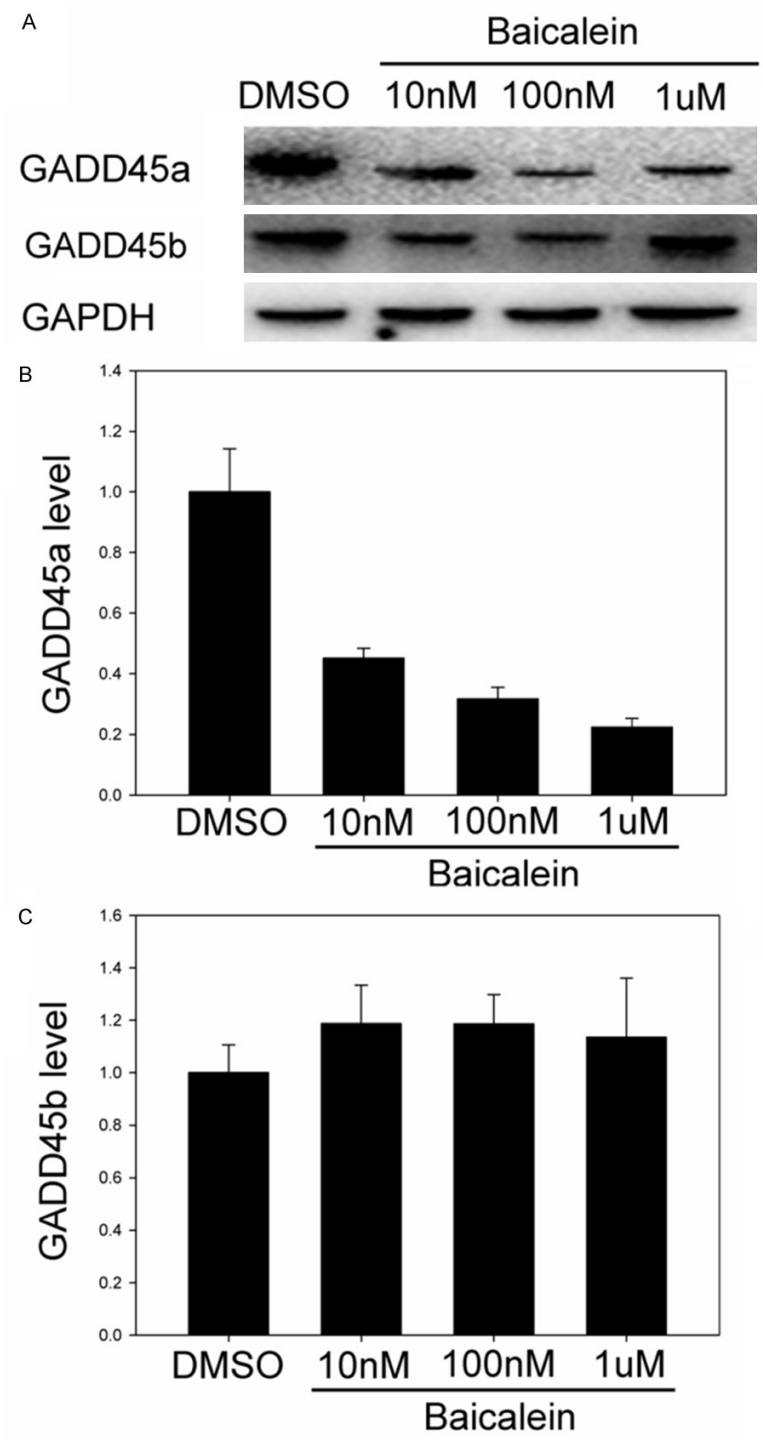

Figure 4. Baicalein reduces the expression of GADD45a in protein and mRNA levels. A. The protein expression of GADD45a and GADD45b in the presence of indicated concentration of baicalein. B, C. The mRNA level of GADD45a and GADD45b in the presence of indicated concentration of baicalein. *P < 0.05, **P < 0.01.

measured in protein level. Result showed that the expression of GADD45a was markedly induced in A549 cell transferred recombinant plasmid (**Figure 5A**). Because of administration of baicalein reduced the expression of GADD45a, the over-expressed effect of GADD45a on baicalein-treated cell was analyzed. Consistent with above result, baicalein-treated A549 cell had a lower proliferation in contrast to DMSO-treated group, however, baicalein-treated A549 cell over-expressed GADD45a prevented the decreased proliferation (**Figure 5B**), thereby revealing the specific ability of restored proliferation for GADD45a. Moreover, we further explored the effect of over-expression of GADD45a on cell cycle distribution of baicalein-treated A549 cell. As expected, A549 cell over expressing GADD45 aprevent-ed baicalein-induced delay in the G1 transition (P < 0.05, **Figure 5C**). These observations indicate that GADD-45a may be an important down-stream signal molecule for baicalein in lung cancer cell.

Discussion

Despite numerous attempts, lung cancer still results in more than 1,000,000 deaths worldwide annually [1, 2]. In part, this is because therapy targeting tumor cells have largely failed. In recent, a line of evidences has indicated that baicalein possesses the powerful anticancer action [24, 25], and baicalein was also applied to induce the cell death in lung cancer cell [13]. However, the detailed molecular mechanism, which accounts for the cells to apoptosis induced by baicalein, was non-elucidated. In present study, we

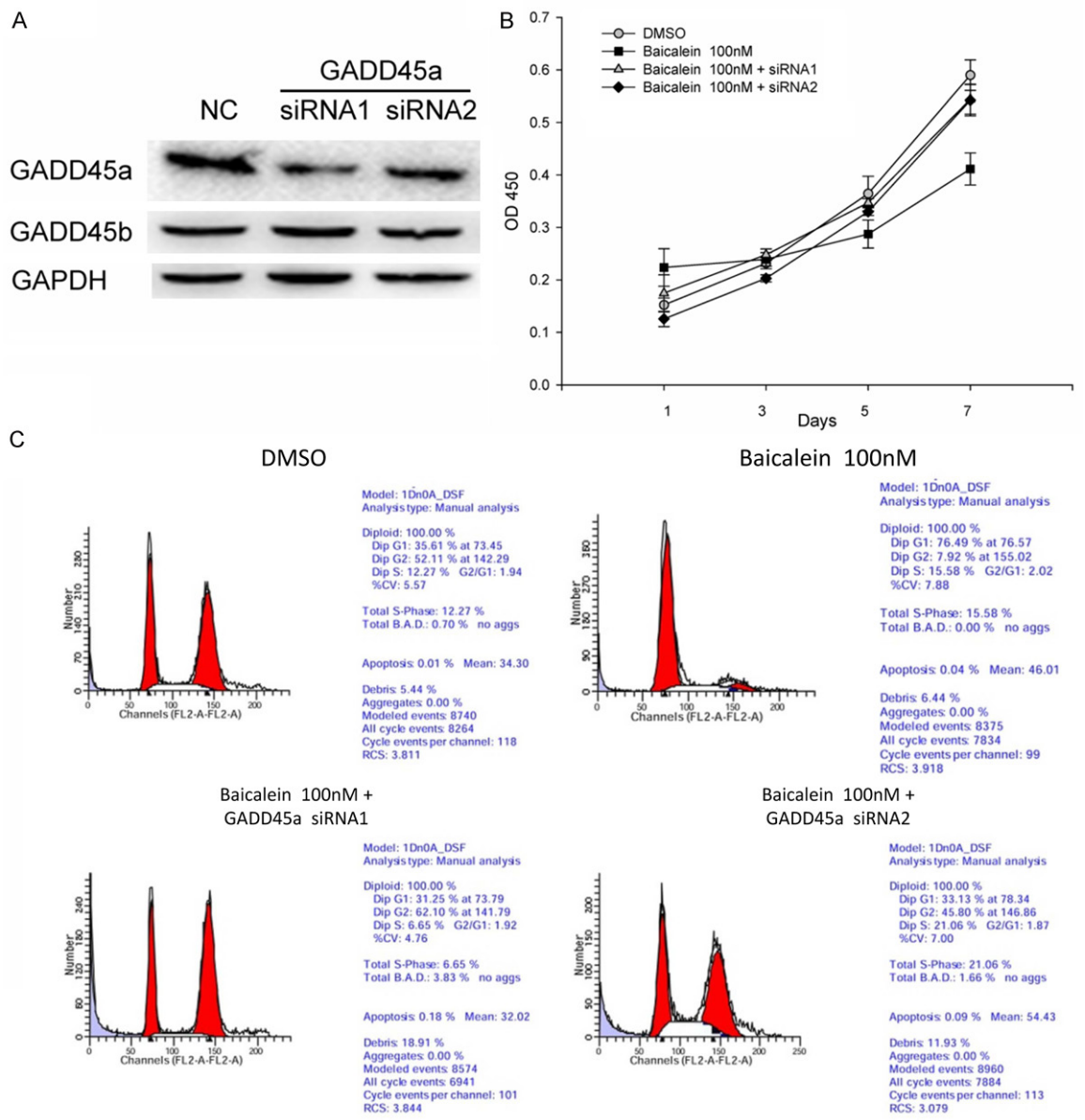

Figure 5. The loss of function of baicalein when over-expression of GADD45a in A549 cell. A. The expression analysis of GADD45a in protein levels in A549 cell treated with pcDNA3-DNMT3a by Western blot. Empty vector was used as negative control in this experiment. B. Over expression of GADD45a enhance the proliferation of baicalein-treated cell. C. Over expression of GADD45a prevents the effect of baicalein on cell cycle distribution of A549. FACS histograms showed a number of cells per channel (vertical axis) vs. DNA content (horizontal axis). $*P < 0.05$, $**P < 0.01$.

not only enrich the baicalein as an anticancer reagent that inhibits proliferation and promotes cell cycle arrest in human NSCLC A549 cell line but also, in follow-up experiments, provide the mechanistic links between baicalein and its anticancer function in lung cancer.

The first core finding in our study is that lower proliferation in baicalein-treated A549 cell was accompany by the down-regulated expression of CDK4, Ki67 and PCNA. CDK4 is a member of cyclin-dependent kinase family and involve in cell cycle G1 phase progression [26]. Continued activation of CDK4-associated kinase is required to maintain tumorigenesis [27]. PCNA is a cofactor of DNA polymerase delta, thereby playing an essential role in DNA replication [28]. The activation of PCNA occurs frequently in breast cancer and can be used as a biomarker of aggressive breast cancer [29]. The Ki-67 protein, also termed as MKI67, is a cellular marker for cell proliferation and early breast

cancer [30, 31]. In addition, CAK4, PCNA and Ki-67 were also found to be highly expressed in lung cancer [32, 33]. These studies reveal that highly expressed CAK4, PCNA and Ki-67 may facilitate the development of lung cancer through elevating the cancer cell proliferation. Given that uncontrolled cell proliferation is the hallmark of malignant tumors, a suitable possibility is that anti-lung cancer activity induced by baicalein is at least partly completed by suppressing the expression of CAK4, PCNA and Ki-67.

Normally, GADD45a, one member of GADD45 family, mediates the cell cycle progression, cell survival and apoptosis [34]. During tumor development, existing evidence supports an intriguing concept that function of GADD45a may be both tumor suppressive and oncogenic. In breast cancer, absence of GADD45 accelerates tumor formation through the inactivation of JNK and p38 kinase, revealing the tumor suppressive function of GADD45a [35, 36]. In pancreatic cancer, GADD45a is highly expressed, but loss of GADD45a expression limits growth and survival of pancreatic ductal adenocarinoma cell, and results in apoptosis and cell cycle arrest in pancreatic cancer cell [37, 38]. Given that, we speculate that dual action of GADD45a may be determined by different cell type, and, based on our outcomes, GADD45a may be oncogenic in lung cancer. More importantly, using RNA-seq, we found that GADD45a is a central downstream target of baicalein. Exogenous baicalein inhibit the expression of GADD45a and ultimately to reduce the A549 cell proliferation, which is supposed to be a novel finding because previous studies did not reveal the relationship between GADD45a and baicalein-induced anticancer activity in lung cancer.

In summary, our work primarily reveals that down-regulated expression of CAK4, PCNA and Ki-67 may be responsible for baicalein-induced anticancer activity, and then provides strong evidence that cell cycle arrest and lower cell proliferation induced by baicalein seems to depend largely on reduction of GADD45a expression. Targeting GADD45aexpression may open up a new avenue for modulation or therapeutic intervention of lung cancer.

Disclosure of conflict of interest

None.

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