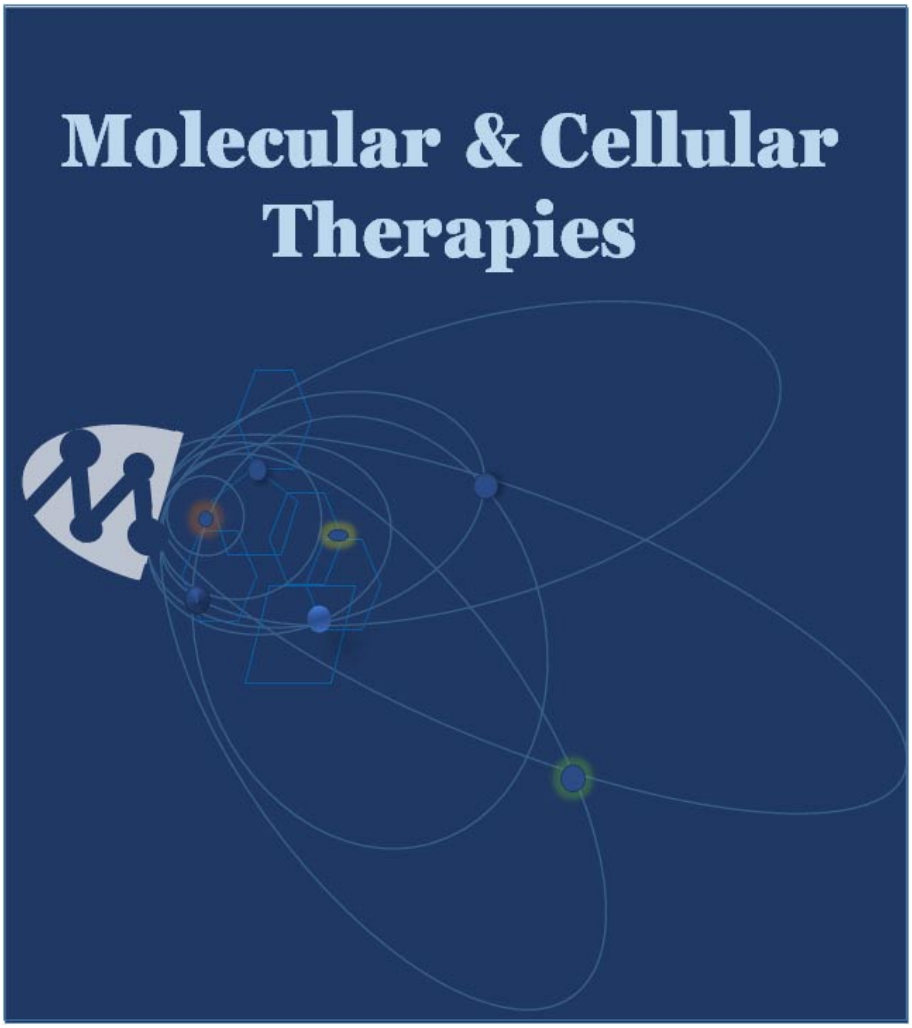


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# Original Research

# Inhibitory effects of Actinidia Chinensis planch root extracts (acRoots) on human lung cancer cells through retinoic acid receptor beta

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## Abstract

**Actinidia Chinensis Planch roots (acRoots) are used to treat many cancers, although the anti-tumor mechanism by which acRoots inhibit cancer cell growth remains unclear. The present study aims at investigating inhibitory effects of acRoots on human lung cancer cells and potential mechanisms. Our data demonstrate that the inhibitory effects of acRoots on lung cancer cells depend on genetic backgrounds and phenotypes of cells. We furthermore found the expression of metabolism-associated gene profiles varied between acRoots-hypersensitive (H460) or hyposensitive lung cancer cells (H1299) after screening lung cancer cells with different genetic backgrounds. We selected retinoic acid receptor beta (RARB) as the core target within metabolism-associated core gene networks and evaluated RARB changes and roles in cells treated with acRoots at different concentrations and timeframes. Hypersensitive cancer cells with the deletion of RARB expression did not response to the treatment with acRoots, while RARB deletion did not change effects of acRoots on hyposensitive cells. Thus, it seems that RARB as the core target within metabolism-associated networks plays important roles in the regulation of lung cancer cell sensitivity to acRoots.**

## Keywords

Actinidia Chinensis Planch roots, lung cancer, RARB, metabolism

## Background

Actinidia Chinensis Planch roots (acRoots) as one of Chinese traditional medicine has therapeutic roles in detoxification, blood swelling, or stroke. Of 60 types, Actinidia Chinensis Planch is a liana plant and grows in temperate climate zones. Seven chemical components were identified from the root of acRoots, including 2alpha-hydroxyoleanolic acid, 2alpha-hydroxyursolic acid, euscaphic acid, 23-hydroxyursolic acid, 3beta-O-acetylursolic acid, ergosta4, 6, 8, 14, 22-tetraen-3-one, and beta-steriol [1]. acRoots is used to treat cancers in stomach, lung, nasopharynges, liver, or colon [2-4], although the mechanism by which acRoots inhibit cancer cell growth remains unclear.

Liver cancer cell sensitivity was found to be associated with cellular immunity and inflammation gene clusters, of which prostaglandin E receptor 3 was as a key regulator of gene expression in response to acRoots [4]. The present study evaluates the inhibitory effects of acRoots on lung cancer cells with different genetic backgrounds and measured dynamic alterations of metabolism-associated gene clusters after the treatment with acRoots at various concentrations. We define metabolism-associated gene networks and the core target within the network of lung cancer cells hypersensitive or hyposensitive to acRoots and investigate the potential mechanism of acRoots inhibition.

## Methods

### Cell source and culture

Lung cancer cells with different genetic backgrounds, e.g. NCI-H358, NCI-H460, NCI-H661, NCI-H1299, NCI-H1650, A549, SPC-A1, or HBE135-E6E7, were provided by cell bank of Shanghai Institutes for Biological Sciences (Shanghai, China). NCI-H358 is non-small cell lung cancer with complete homozygous deletion of the p53 gene and with 2240 mutations and 60 genes with altered copy number. NCI-H460 is large cell lung carcinoma with about 700 mutations and 450 genes with altered copy number. NCI-H661 is large cell lung cancer with 1600 mutations and 2500 genes with altered copy number. NCI-H1299 is non-small cell lung cancer with 980 mutations and 2000 genes with altered copy number. NCI-H1650 is lung adenocarcinoma with 320 mutations and 430 genes with altered copy number. A549 is a lung adenocarcinoma with the KRAS mutation (p.G12Sc.34G>A), 710 mutations, and

210 genes with altered copy number. SPC-A1 is a lung adenocarcinoma without gene copy number variation. HBE135-E6E7, derived from normal bronchial epithelium taken from a man with squamous cell carcinoma, express high levels of mRNA for epidermal growth factor receptor (EGFR), transforming growth factor-alpha (TGF-alpha), and amphiregulin (AR), but not epidermal growth factor (EGF). All cells were cultured in RPMI 1640 medium (Biowest, France) supplemented with 10% inactivated fetal bovine serum (Biowest), under certain circumstances of humidified air at 37°C with 5% $CO_2$.

**acRoots preparation**

acRoots were mashed and mixed with tenfold double distilled water, with 100°C heat for an hour. After 2 cycles of procedures of filtration, the extract was concentrated into 1g/ml ultimately.

**Cell screening**

NCI-H358, NCI-H460, NCI-H661, NCI-H1299, NCI-H1650, A549, SPC-A1 and HBE135-E6E7 ($1\times10^3$/well) were seed in growth media 96-well plates. After 24 hours incubation, cells were treated with acRoots at concentrations of 1, 3, 5, 10, 20, 30, 40, 50, or 100 mg/ml for 24, 48 or 72 hours. Cells were then treated with 10μl CCK8 (Dojindo, Japan)/ well with 100μl of fresh RPMI 1640 and incubated for 4 hours at 37°C. The number of viable cells were measured by absorbance at 450nm using FlexStation 3 multi-mode microplate reader (Molecular Devices, USA).

**Measurement of gene expression profiles**

NCI-H460 and NCI-H1299 were chosen as acRoots-hypersensitive and hyposensitive representative after the screening and selection for microarray. NCI-H460 expresses easily detectable p53 mRNA, but NCI-H1299 lacks expression of p53 protein. Microarray experiments were performed as former described [5]. Briefly, total RNA was amplified and labeled by Low Input Quick Amp Labeling Kit, One-Color (Agilent technologies, Santa Clara, CA, US). Labeled cRNA were purified by RNeasy mini kit (QIAGEN, GmBH, Germany). Each slide was hybridized with 1.65μg Cy3-labeled cRNA using Gene Expression Hybridization Kit (Agilent technologies, Santa Clara, CA, US) in Hybridization Oven (Agilent technologies, Santa Clara, CA, US). After 17 hours hybridization, slides were washed in staining dishes (Thermo Shandon, Waltham, MA, US) with Gene Expression Wash Buffer Kit (Agilent technologies, Santa Clara, CA, US) and scanned by Agilent Microarray Scanner (Agilent technologies, Santa Clara, CA, US) with default settings. Data were extracted with Feature Extraction software 10.7 (Agilent technologies, Santa Clara, CA, US). Raw data were normalized by Quantile algorithm, Gene Spring Software 11.0 (Agilent technologies, Santa Clara, CA, US).

**Validation of the Microarray Genes**

After the bioinformatics analysis, the selected genes were validated by NCI-H358, NCI-H460, NCI-H661, NCI-H1299, NCI-H1650, A549, SPC-A1 and HBE135-E6E7 cells. The cells ($5\times10^4$/well) were seed in 24-well plates for 24 hours incubation and then treated with acRoots at 10mg/ml for 48 hours. After the termination, total RNA was prepared with TRIzol reagent (Invitrogen, USA). Complementary DNA was synthesized using reverse transcription reagent kit (TaKaRa, Japan) from 1μg of total RNA. Real-time PCR was performed using the following primers by PrimeScript ® RT reagent Kit (TaKaRa). Amplification and detection of mRNA were analyzed by 7500 real-time PCR System (Applied Biosystems, USA). The relative amount of gene was normalized against the glyceraldehyde-3-phosphate dehydrogenase mRNA. All real-time PCRs were performed in triplicate, and the changes in gene expression were exhibited as fold-increases relative to untreated controls.

**Cell transfection and RNA interference**

After the selection and validation in NCI-H358, NCI-H460, NCI-H661, NCI-H1299, NCI-H1650, A549, SPC-A1 and HBE135-E6E7 cells, RARB gene was chosen as the core target within metabolism-associated networks and transfected with small interference RARB RNA in H460 cell. After 24 hour incubation, cells were treated with RARB-SiRNA (RARB-Homo-999: GACGAUCUCACAGAGAAGATT, UCUUCUCUGUGAGAUCGUCTT; RARB-Homo-1140: GCCACCAAGUGCAUUAUUATT, UAAUAAUGCACUUGGUGGCTT; RARB-Homo-1245: GACAUCCUGAUUCUUAGAATT, UUCUAAGAAUCAGGAUGUCTT; RARB-Homo-1666: CACCUCUCAUUCAAGAAAUTT, AUUUCUUGAAUGAGAGGUGTT) and NC (Negative control: UUCUCCGAACGUGUCACGUTT, ACGUGACACGUUCGGAGAATT) (GenePharma, China) at 0.01, 0.1, or 1.0 umol/L. Lipofectamine 2000 transfection reagent (Invitrogen, USA) at 2ul/ml per well was used for 6 hours and changed to the complete cell culture medium for 24, 48 or 72 hours. Total RNA was prepared with TRIzol reagent and complementary DNA was synthesized using reverse transcription reagent kit (TaKaRa, Japan) from 1μg of total RNA to measure gene expression of RARB. After screening out the optimal cell transfection and RNA interference condition, cells were treated with acRoots at 0, 5, 10, or 30 mg/ml for 24 hours to detect cell proliferation and gene expression of RARB.

## Results

**Inhibitory effects of acRoots on lung cancer cells**

The cell sensitivity to the drug was screened and evaluated using concentrations of acRoots from 1 to 100 mg/ml and dynamically from 24 to 72 hours. Of those cells, H1299 cell proliferations were significantly inhibited from 5 mg/ml of acRoots with a concentration-dependent pattern (Figure 1A). SPC-A1 cell significantly increased from 3 mg/ml (Figure 1B). HBE cells became hypersensitive to acRoots from 3 mg/ml and died from 5 mg/ml (Figure 1C). Significant inhibition of acRoots was noticed in A549 (Figure 1D), H661 (Figure 1E), or H358 cells (Figure 1F) at 30 mg/ml. The proliferations of H460 (Figure 1G) and H1650 (Figure 1H) cells were significantly

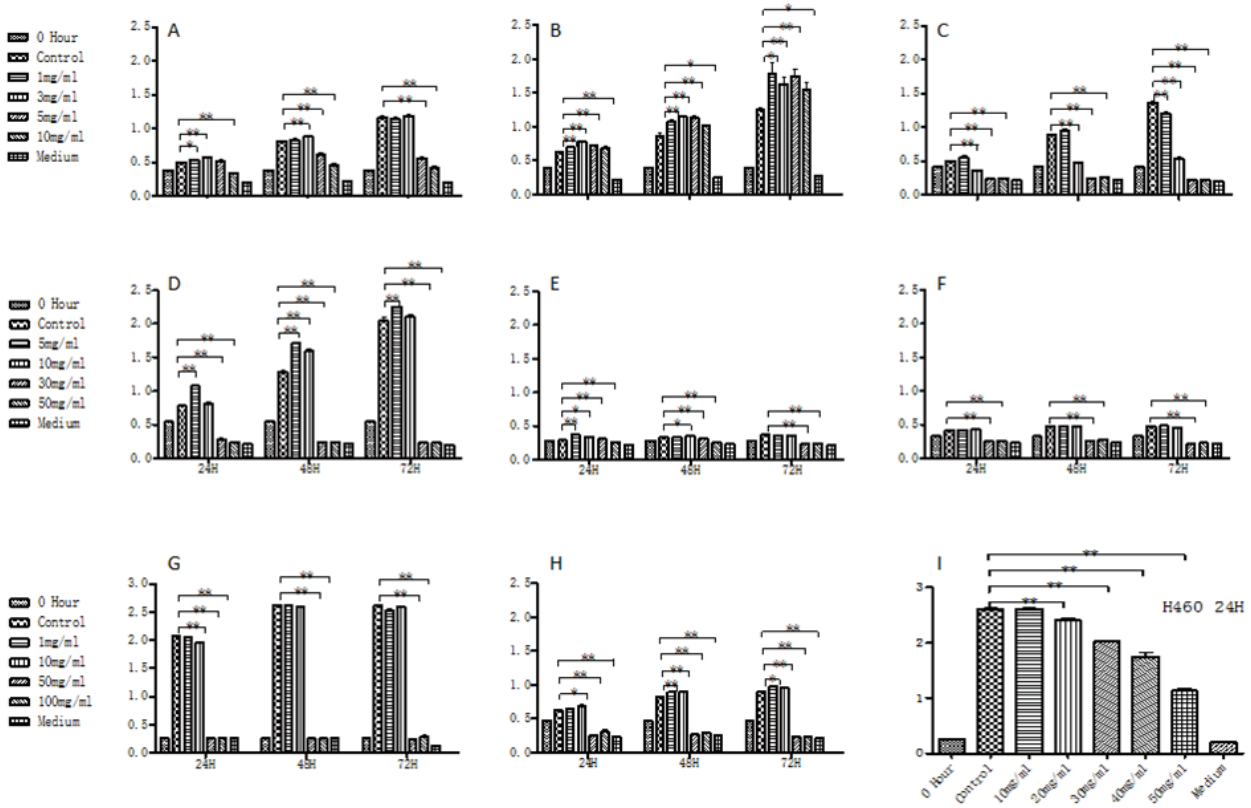


Figure 1: The cell sensitivity to acRoots was screened and evaluated using concentrations of acRoots from 1 to 100 mg/ml and dynamically from 24 to 72 hours. Of those cells, H1299 (A), SPC-A1 (B), and HBE (C) cells were treated with acRoots at 1, 3, 5, or 10 mg/ml, A549 (D), H661 (E), or H358 (F) cells at 5, 10, 30, or 50 mg/ml, and H460 (G) and H1650 (H) cells at 1, 10, 50, or 100 mg/ml. H460 cells were further treated with acRoots at 10, 20, 30, 40, or 50 mg/ml for 24 hours (I). * and ** stand for p values less than 0.05 and 0.001, respectively, as compared with controls.

inhibited by acRoots from 50 mg/ml. To further evaluate the concentration window, H460 cells were treated with acRoots at 10, 20, 30, 40, or 50 mg/ml for 24 hours and showed hypersensitive to the drug from 20 mg/ml in a concentration-dependent pattern (Figure 1I). Thus, H1299 cells were selected as an acRoots-hypersensitive representative, while H460 as hyposensitive one.

**Expression of metabolism-associated gene clusters**

The expression of about 39888 genes was changed in H1299 and H460 cells after the treatment with acRoots (Supplement 1). The expression of metabolism-associated gene clusters in H460 cell was shown in Figure 2A, of which some were continuously up-regulated (Figure 2B) or down-regulated (Figure 2C). We summarized eight dynamic change pattern in Figure 2D where the number of genes in each pattern were listed. Figure 2E showed top 10 of genes in the mode of consistent down-regulation as an example. Of those selected genes, interleukin 21 receptor (IL21R), putrescine/proton symporter ankyrin domain family member D (POTED), and nuclear protein 1 (NUPR1) were selected and furthermore validated in different lung cancer cells. Our results indicate some of selected genes were altered in the same pattern as microarray showed (Figure 2F), even though the same gene probes were used.

Figure 3A showed the alterations of genes in H1299 cells after treatment with acRoots at 1, 5, or 10 mg/ml. The eight change patterns of metabolism-associated genes with gene numbers were presented in Figure 3E. Of those, 7 genes consistently increased (Figure 3B), 8 or 24 genes similarly decreased between 1 and 5 mg/ml (Figure 3C) or between 5 and 10 mg/ml (Figure 3E), or 60 genes decreased maximally from 1 mg/ml (Figure 3F). Three selected genes, e.g. heat shock protein family A member 6 (HSPA6), discoidin domain receptor (DDR), and G protein-coupled receptor 12 (GPR12) were further validated in different cells. We noted the expression of selected genes in most of cells reduced, while HSPA6 increased in HBE, H1650, or H1299 (Figure 3G).

**Selection of the core target within networks**

Different concentrations and treatment schedule were selected between H460 and H1299 cells, after the screening of acRoots inhibitory effects (Figure 4A). The distribution of changed genes in H460 (Figure 4B) or H1299 cells (Figure 4C) demonstrated the same pattern. Of those genes, the expression of RARB, glutamyl-tRNA amidotransferase binding protein 4 (GATA4), lysozyme like 4 (LYZL4), and suppression of tumorigenicity 8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 3 (ST8SIA3) significantly decreased at H460 and H1299 cells (Figure 4D). The chromodomain helicase DNA binding

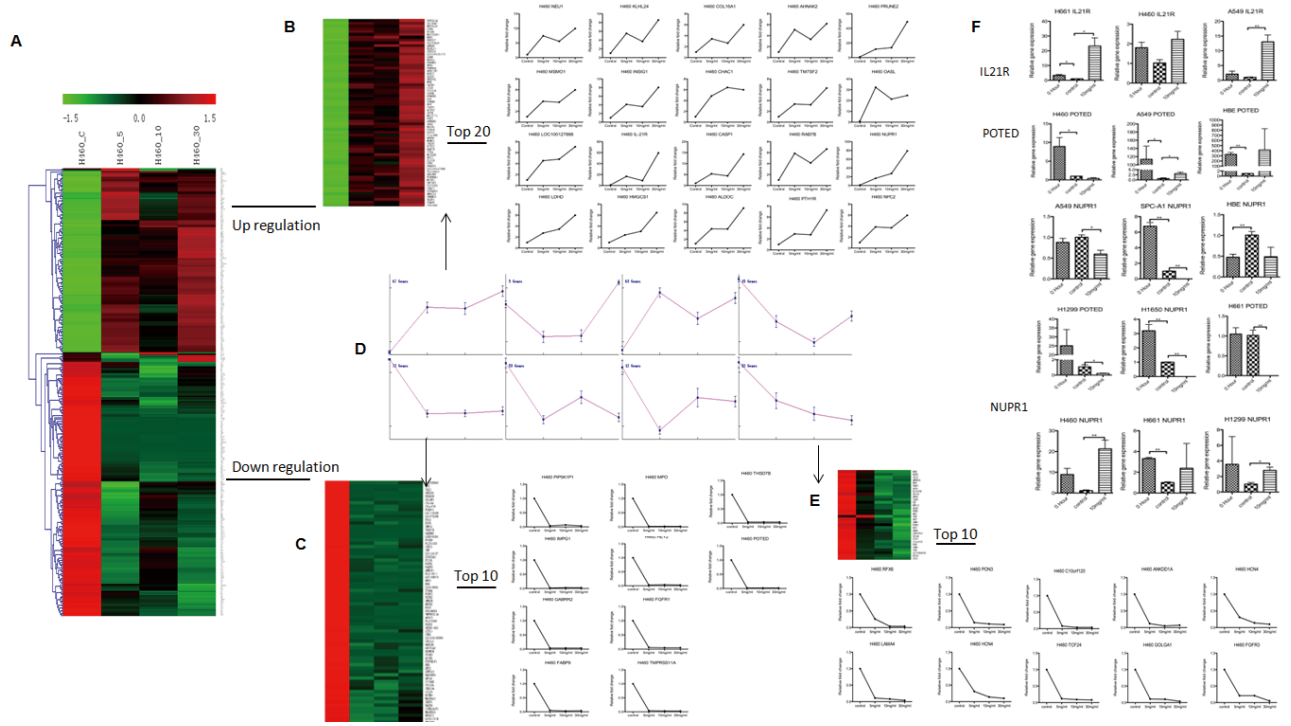


Figure 2. Hierarchical clusters of metabolism-associated genes (A) in hyposensitive H460 cells treated with acRoots at 0, 5, 10, or 30 mg/ml for 24 hours. Of those, top 20 of up-regulated (B) or top 10 down-regulated genes (C) were selected from the consistent up- or down-regulated clusters of eight gene change patterns (D). A cluster of genes gradually down-regulated in a concentration-dependent pattern (E). As selected elements from the screening, the expression of interleukin 21 receptor (IL21R), putrescine/proton symporter ankyrin domain family member D (POTED), and nuclear protein 1 (NUPR1) genes were validated in H460 cells immediately (0h) and 24 hours after treatment with vehicle (Control) or acRoots at 10 mg/ml. * or ** stand for p values less than 0.05 or 0.01, respectively, as compared with Control.

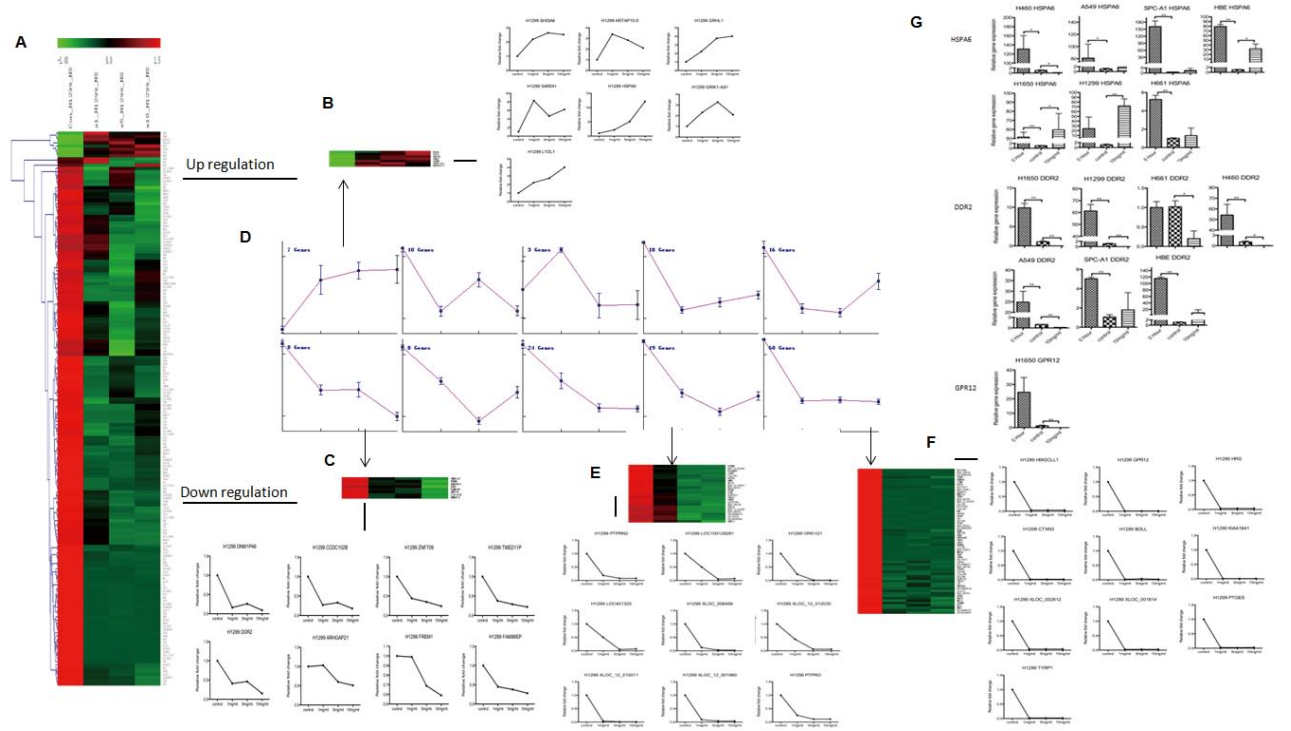


Figure 3. Hierarchical clusters of metabolism-associated genes (A) in hyposensitive H1299 cells treated with acRoots at 0, 1, 5, or 10 mg/ml for 24 hours. Of those, up-regulated (B) or down-regulated genes (C) were selected from the consistent up- or down-regulated clusters of eight gene change patterns (D). Two clusters of genes rapidly down-regulated in a concentration-dependent pattern (E,F). As selected elements from the screening, the expression of heat shock protein family A member 6 (HSPA6), discoidin domain receptor (DDR), and G protein-coupled receptor 12 (GPR12) genes were validated in H460 cells immediately (0h) and 24 hours after treatment with vehicle (Control) or acRoots at 10 mg/ml. * or ** stand for p values less than 0.05 or 0.01, respectively, as compared with Control.

protein 5 (CHD5) and protein tyrosine phosphatase-receptor type N2 (PTPRN2) genes increased in H460 cells, while the glutamate ionotropic receptor kainate type subunit 1antisense RNA 1 (GRIK1-AS1) gene was up-regulated in H1299 cells, while down-regulated in H460. The validation of RARB in different cells demonstrates that the expression of RARB gene was significantly down-regulated in most of lung cancer cells (Figure 4E, $p<0.05$ or 0.01, respectively, *vs* Controls), except for HBE.

Of metabolism-associated networks and dynamic networks of gene interactions of different lung cancer cells treated with acRoots at different concentrations, there are 20 genes to form the core network, including Nr2c1, Nr2e1, Rxrg, Nr4a2, Vdr, Rara, Thrb, Ncoa3, Esr2, Esr1, Rxra, Nrip1, Hdac1, Sp1, Ncoa1, Src, Ep300, Ncoa2, Brca1, Rarb, and Med1. Most of those genes were closely associated and interacted with RARB. String Network analysis (www.string-db.org) of the relationships of RARB demonstrated direct (physical) and indirect (functional) associations or interactions of the closely metabolism-associated and inhibition-selected 20 genes (Figure 5A). We found that concentration-associated expressions of selected genes were not consistent with RARB gene changes (Figure 5A). Of those selected genes within network, HDAC1, SRC, NCOA1, NCOA2, NCOA3, and EP300 were found in one signaling pathway-thyroid hormone signaling pathway (Figure 5B).

**Validation of RARB-dominant mechanisms**

RARB-dominant signaling pathway demonstrates that most of associated factors in the RARB-driven mechanism have the similar change patterns, even though the patterns were different from RARB one (Figure 6). H460 cells have the highest expression of RARB gene without acRoots, as compared with other lung cancer cells, and A549 and H1299 cells were the second high expression of RARB which is about one third of H460 (Figure 7A). In order to define the sensitivity of H460 RARB expression to challenges, inflammatory stimulus lipopolysaccharide (LPS) from Salmonella typhi used in the present study could slightly up-regulate RARB expression at the early stage (4 and 8 hours) after the challenge (Figure 7B), while significantly down-regulate RARB from 24 and on in a dose-dependent pattern (Figure 7C). LPS at dose of 1.0 μmol down-regulated RARB expression almost 100% at 24 hours, while about 60% at 72 hours. Designed GAPDH gene small interfering RNA (siRNA) in H460 cells has the interference efficiency over 95% (Figure 7D) to validate the process of siRNA synthesis.

We furthermore designed four RARB siRNA targeting sequences and found that the interference efficiency of RARB siRNA-1140 in H460 was higher than 80% (Figure 8A). The combination of acRoots and RARB siRNA could turn off the expression of RARB genes 24 hours after the treatment. The proliferation of H460 cells significantly increased after the deletion of RARB gene, as compared with cells without deletion or with sham small interference (Figure 8B). The most important finding was that the proliferation of H460 with RARB siRNA significantly decreased from 24 hours and on after acRoots at 5 mg/

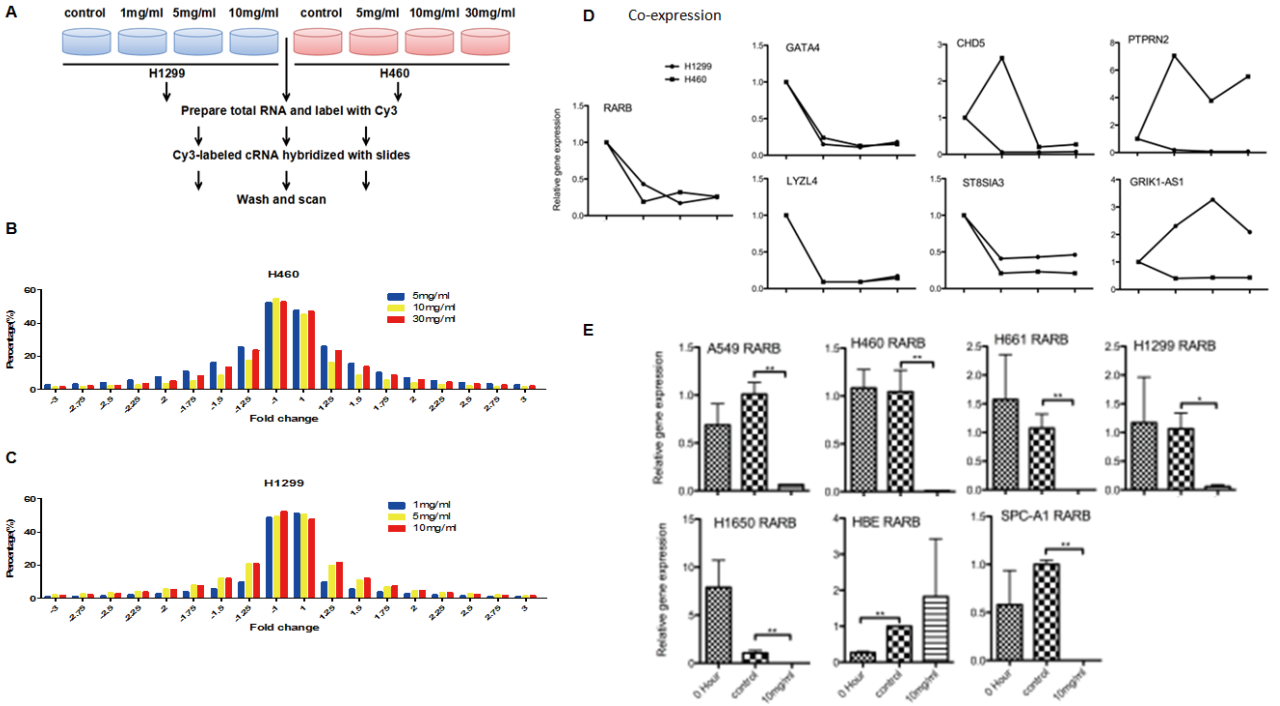


Figure 4. In the experimental design (A), the distribution of changed clusters in H460 (B) or H1299 cells (C) showed the different patterns, of which some of metabolism-associated genes co-expressed in both cells after treatment with acRoots at different concentrations (D). RARB was selected as the core target within RARB-driven networks and further validated in lung cancer cells with different genetic backgrounds (E). * or ** stand for p values less than 0.05 or 0.01, respectively, as compared with Control.

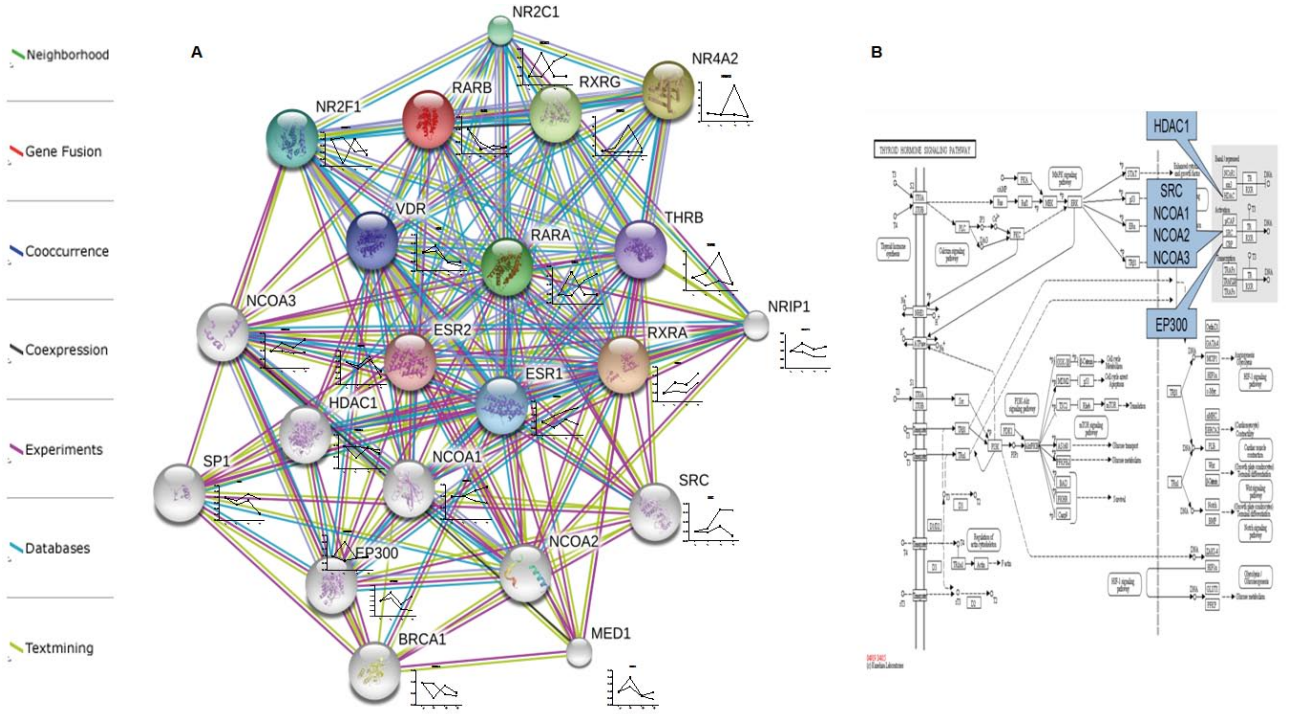


Figure 5. RARB-driven metabolism-associated networks (A) and potential signal processes (B). Inhibitory effects of acRoots in each element within the network were labelled. NR2C1: nuclear receptor subfamily 2 group C member 1, NR4A2: nuclear receptor subfamily 4 group A member 2, NR2F1: nuclear receptor subfamily 2 group F member 1, RARB: retinoid acid receptor beta, RXRG: retinoid X receptor gamma, NCOA3: nuclear receptor coactivator 3, VDR: vitamin D receptor 3, RARA; retinoic acid receptor alpha, THRB: thyroid hormone receptor beta, ESR2: estrogen receptor 2, RXRA: retinoid X receptor alpha, NRIP1: nuclear receptor interacting protein 1, ESR1: estrogen receptor 1, SP1: specificity protein 1 transcription factor, DAC1: histone deacetylase 1, NCOA1: nuclear receptor coactivator 1, SRC: sarcom proto-oncogene, non-receptor tyrosine kinase, NCOA2: nuclear receptor coactivator 2, EP300: histone acetyltransferase p300, BRCA1: breast cancer 1, mediator complex subunit 1.

ml, as compared with cell with siRNA but not acRoots ($p<0.05$). Increased sensitivity of H460 cells without RARB gene showed acRoots concentration-dependent and time-dependent.

The cytochrome P450 monooxygenases (CYP) and its isoforms are the primary retinoid-inactivating enzyme and regulates the embolism. We found that the expression of CYP26A1 (Figure 8C) and CYP2E1 (Figure 8D) did not change, while CYP26B1 (Figure 8E) increased after treatment with acRoots at 5, 10, or 30 mg/ml. The deletion of RARB gene could completely down-regulate the expression of CYP26A1 and CYP26B1, while up-regulate CYP2E1 about 5 folds over cells with RARB. Those RARB siRNA-induced changes were reserved with acRoots from 5 mg/ml.

## Discussion

Our results show inhibitory effects of acRoots in lung cancer cells with different genetic backgrounds, which are associated with the variation of sensitivities to the drug. The anti-tumor effect of acRoots was suggested to be associated the activation of the p38 mitogen-activated protein kinase, Caspase9, or poly-(ADP-ribose) polymerase signal pathways, the induction of apoptosis, or cell death [6]. The inhibitory effects of acRoots in lung cancer cells was due to the cytotoxic activity of phenolic elements from acRoots [2]. There is little to know how lung cancer cells develop various sensitivities to acRoots and what the critical limiting factors are in the regulation of cell susceptibility. Study on kiwifruit gene sequencing, structure, and transcript abundance at developmental stages demonstrated that critical genes are mainly involved in the biosynthesis and metabolism of elements, especially in sucrose and starch metabolism [7]. It indicates that metabolism-associated genes play important roles in the development of kiwifruit and trucks.

We hypothesize that acRoots interact with metabolism-associated genes and pathways in cancer cells reciprocally to inhibit cancer cell growth and metastasis. The previous studies from our group demonstrated that acRoots could influence the expression of cholesterol metabolism-associated genes in hepatocellular carcinoma [8]. acRoots could influence cholesterol synthesis and uptake of cancer cells, probably through the activation of proprotein convertase subtilisin/kexin type 9, resulting in the reduction of lipid receptor expression and uptake, total intracellular cholesterol, and cell proliferation.

The present study identified the acRoots-hypersensitive or hyposensitive lung cancer cells on basis of the inhibitory

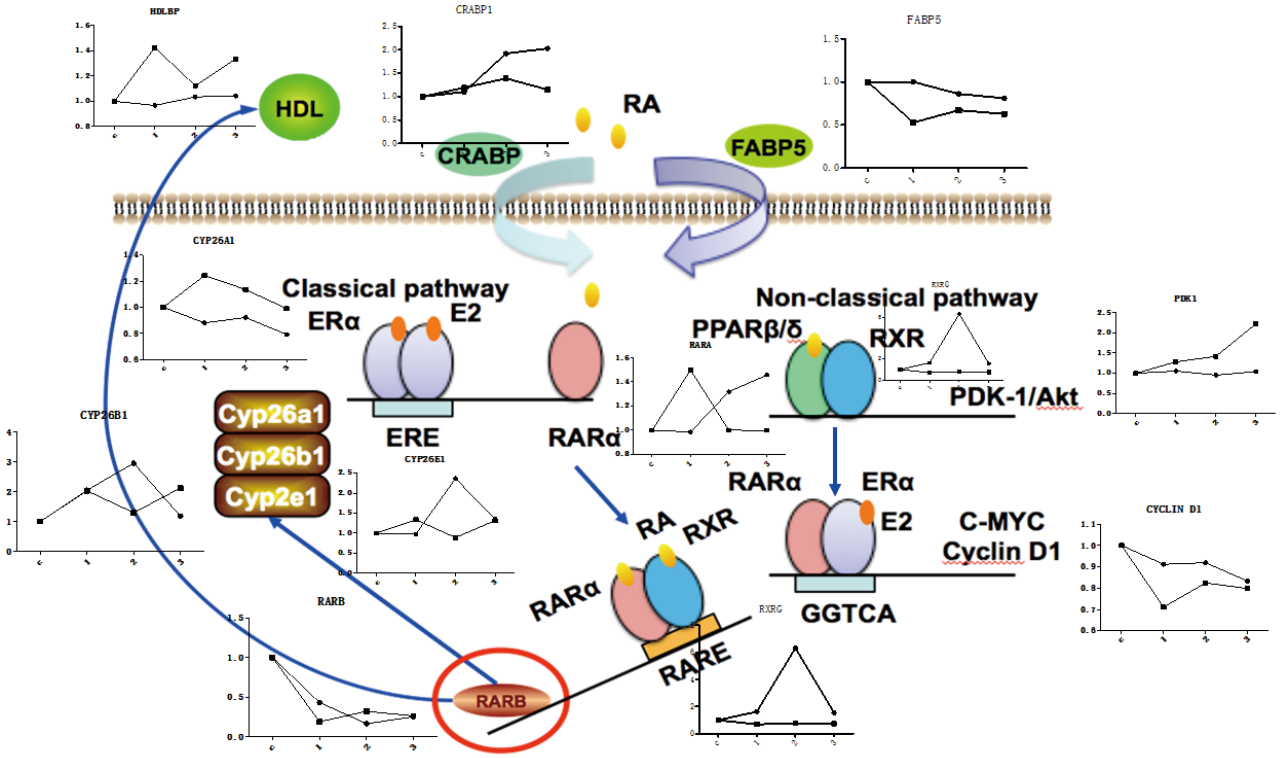


Figure 6. Retinoid acid receptor b (RARB)-dominant molecular mechanisms. The retinoid acid (RA) binds with cellular retinoic acid binding protein (CRABP) or fatty acid binding protein 5 (FABP5), and activates classic pathways with estrogen receptor 1 (ER ) and estrogen 2 (E2) or non-classic pathways with peroxisome-proliferator-activated receptor (PPAR) and retinoid-X-receptor (RXR). E2-responsive elements with ER and E2 gather with RAR were formed for an activated cluster with RXR and RARE, to alter RARB activities. RARB then interacts with cytochrome P450 monooxygenases (CYP) 26A1, 26B1, or 2E1, or with high-density lipoprotein (HDL). Inhibitory effects of acRoots in each element within the network were labelled.

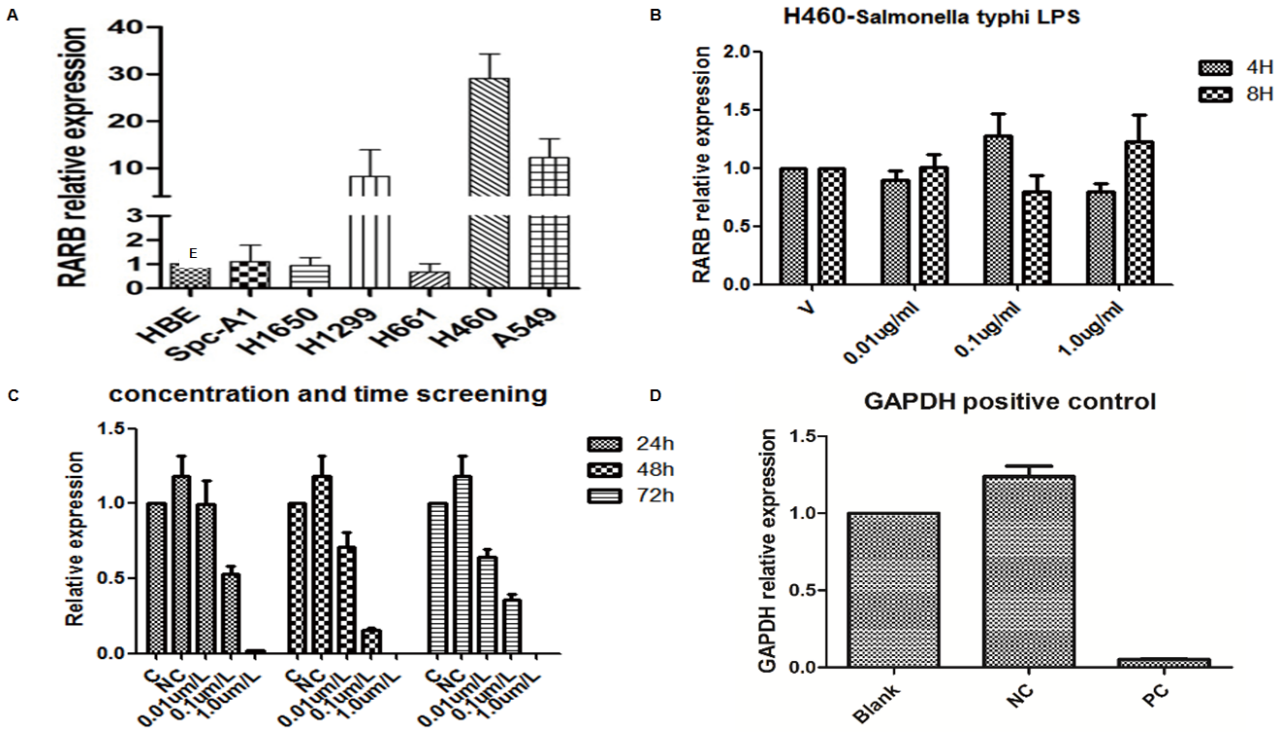


Figure 7. Expression of RARB gene in lung cancer cells with different gene backgrounds (A), and in H460 at the early phase (4 and 8 hours) (B) and 24-72 hours (C) after the challenge with LPS at different concentrations. The methodology to delete RARB gene was validated with RARB siRNA (D) as the positive control (PC), as compared with those with vehicle as the negative control (NC) and without any (Blank).

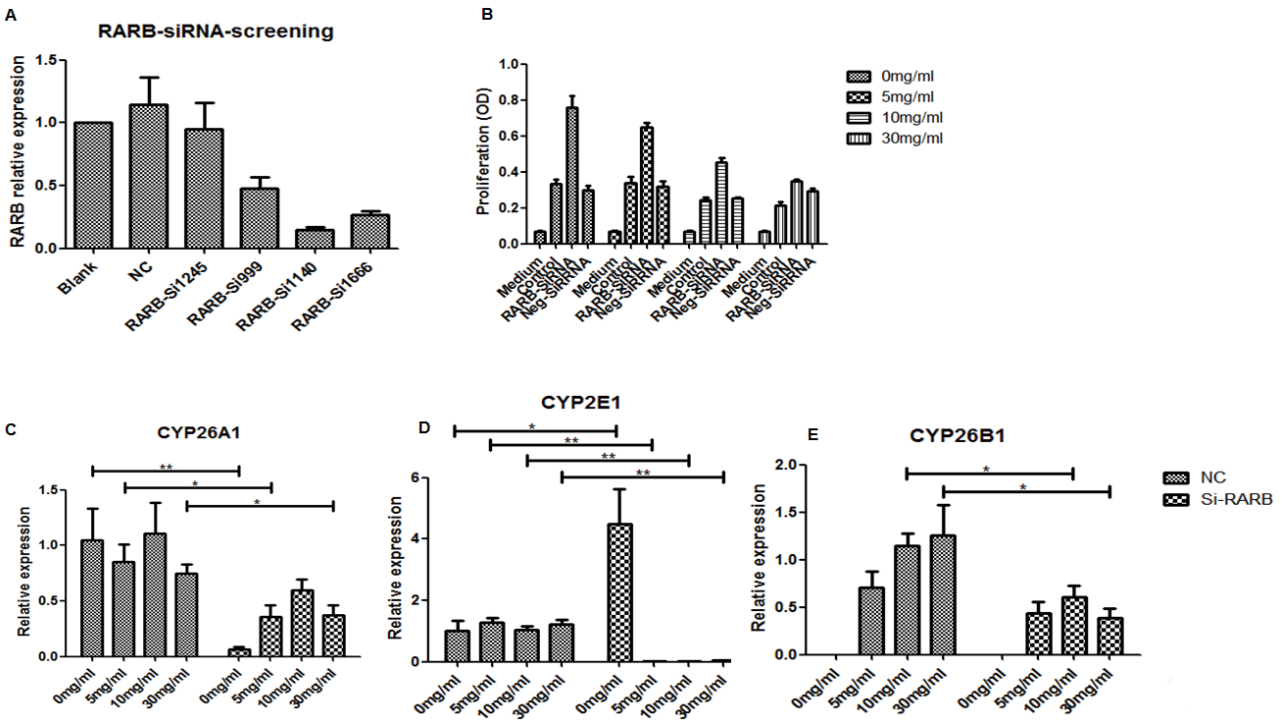


Figure 8. Different RARB siRNA sequences were validated in H460 cells for 24 hours (A), Cell proliferations were measured in H460 cells with medium, vehicle, RARB siRNA, or negative siRNA 24 hours after the treatment with acRoots at concentrations of 0, 5, 10, or 30 mg/ml (B). Expression of CYP26A1 (C), CYP2E1 (D), and CYP26B1 genes (E) was measured in cells treated with vehicle (NC) or RARB siRNA (Si-RARB) 24 hours after treatment with acRoots at concentrations of 0, 5, 10,or 30 mg/ml. * or ** stand for p values less than 0.05 or 0.01, respectively, as compared with Control.

effects in a concentration-dependent pattern, in order to avoid the cytotoxicity of drugs and have a biological window for intervention [9]. We found both H1229 and HBE cells hypersensitive to acRoots and selected H1299 as the hypersensitive representative, since H1299 cells showed a clear concentration-dependent inhibition, high malignance, and more gene mutations, as compared with HBE cells. Among H460, H1650, A549, H661, and H358 cells in response of high concentrations of acRoots, we found that A549 and H1650 cells failed to show the acRoots-concentration-dependent inhibition, though we tried to different concentrations of acRoots between 10 and 30 mg/ml. H661 and H358 cells have the low capacity of proliferation and a small window between with or without effects. As the hyposensitive representative, H460 cells a clear acRoots concentration-dependent inhibition between 10 and 50 mg/ml at 24 hours after the treatment. More investigations are to be needed to furthermore clarify the specificity of H1299 cell hypersensitivity or H460 cell hyposensitivity to acRoots.

The heterogeneity of cell sensitivity to drug is highly dependent genetic factors. The gene expression, mutation, and epigenetics are associated with a large range of efficacy, adverse reactions, or toxicity and account for the hypersensitivity and hyposensitivity seen in different patients [10]. The present study at the first time investigates the influence of acRoots in gene expression profiles of lung cancer cells to identify the genomic difference between acRoots-hypersensitive and hyposensitive lung cancer cells. We defined the hypersensitivity-specific (e.g. HSPA6, DDR2, GPR12) or hyposensitivity-specific metabolism-associated gene profiles (e.g. IL21R, POTED and NUPR1) as well as co-expression of some genes in both. One strategy to improve the cell sensitivity is to down-regulate or up-regulate hypersensitivity-specific or hyposensitivity-specific genes. However, the present study found that RARB as the most common metabolism pathway was obviously down-regulated in both hypersensitive and hyposensitive lung cancer cells, while cells did have different sensitivities. We propose whether the down-regulation of RARB gene can augment the sensitivity of hyposensitive cells.

RARB is a member of the retinoic acid receptor subfamily, together with others, dominantly regulates the retinoic acid activity through binding specific retinoic acid response elements in target genes promoters. RARB is one of critical ligand-regulated transcription factors to centralize the cancer cell growth and metastasis through the RA and retinoid signaling pathways [11]. RARB was suggested as a potential therapeutic target to protect from solid tumors by the frequent epigenetic silencing of RARB [12]. Comprehensive expression of retinoic acid receptors in non-small cell lung cancer tissue was about up to 60% lower than in the normal [13], while extensive retinoid-based therapies, e.g. synthetic agonists and antagonists of retinoic acid receptors, failed to the expected outcomes

[14], due to poor understanding of mechanisms or RARB-dominant network and interaction.

The integration of the gene expression with gene interactions and networks provides a better view to understand the role of selected core targets in multiple signal pathways. We developed an individualized network model to integrate differential gene expression, variance, and gene-pairs with the differential expression covariance and to define the heterogeneity of disease samples [15]. It is important to define differential gene networks of individuals, differential expression variance/covariance, and expression state or activity of various feature genes and their network or modules for an individual. We explored the transcriptional regulatory networks between subtypes of lung cancer and selected the core elements within networks to drive the force of interactions among modules [16]. In a large number of networks, we identified a network constructed with elements with significant changes after treatment with acRoots and found that RARB is the driver force among interactions and networks in hypersensitive or hyposensitive lung cancer cells. The target genes identified from gene interactions and networks were validated and confirmed as core genes in lung cancer [17]. From the molecular mechanism of retinal acid-RARB-signal pathways, we found the variation of acRoots-altered gene expressions of involved factors, even though those might interact or signal each other. In addition to gene expression, gene epigenetics should be considered, since methylation changes are specific for risk factors in lung cancer and DNA methylation changes are associated with the development of lung cancer [18].

The fact that RARB expression in both hypersensitive and hyposensitive lung cancer cells reduced in acRoots concentration-dependent pattern demonstrates that RARB is strongly associated with inhibitory effects of acRoots. It seems that acRoots inhibit lung cancer cell proliferation through the alterations of lipids and lipid-soluble ligands regulated by nuclear hormone receptors. Our data demonstrate that acRoots inhibited RARB expression and heterodimer formation, resulting in the disconnection with and accumulations of the retinoid X receptor. The dysfunctional interaction between retinoic acid receptors and retinoid X receptors compromises the recognition and responsiveness to retinoic acid, oxysterols, polyunsaturated and oxidized fatty acids, and 1α,25-dihydroxyvitamin.D3 [19]. However, the complete deletion of RARB gene increased the proliferation of hyposensitive lung cancer cells and the cell sensitivity to acRoots. It is possible that other compensative pathways occur when RARB is deleted or blocked, although the exact compensation and mechanism need to be furthermore explored. Another question is whether acRoots-induced down-regulation of RARB is acRoots inhibition-specific or a general reaction of the compensation, since we found that LPS could also induce the down-regulation of RARB gene. It is also critical to validate the repeatability and stability of RARB-interacted elements in metabolism-associated network as efficacy-specific biomarkers and druggable targets [21-26].

RARB has a close communication and interaction with CYP26A1, CYP26B1 and CYP2E1 in the RARB-dominant signaling pathway. Of those, CYP26A1 plays a key role in retinoic acid metabolism and acts with all-trans-retinoic acid, to make 4-hydroxylation and 18- hydroxylation and generate hydroxylated forms of 4-OH-, 18-OH, or 4-oxo-retinoid acid [20]. acRoots elevated the expression of CYP26A1 gene in hypersensitive cells and CYP26B1 in hyposensitive cells, while CYP2E1 in both. The deletion of RARB completely down-regulated the expression of CYP26A1, CYP26B1, and CYP2E1, while acRoots could restore partial expression (about 50-60%) of CYP26A1 or CYP26B1, rather than CYP2E1.

## Conclusions

In conclusion, we firstly screened concentration- or time-dependent inhibitory effects of acRoots in lung cancer cells with different genetic backgrounds to select a hypersensitive (H1299 cells) or hyposensitive representatives (H460 cells). We identified the hypersensitivity- or hyposensitivity-specific metabolism-associated gene clusters and co-expression of genes in both. We explores the interactions and networks of metabolism-associated genes in hypersensitive and hyposensitive cells treated with or without acRoots and defined the RARB-driven network as the core elements. RARB expression in both hypersensitive and hyposensitive lung cancer cells reduced in acRoots concentration-dependent pattern demonstrates. The deletion of RARB gene increased the proliferation of hyposensitive lung cancer cells and the cell sensitivity to acRoots. Thus, our data indicate that the RARB-dominant network and signal plays the important role in the regulation of lung cancer cell sensitivity to drug, although the specificity of RARB-dominant sensitivity needs to be further validated.

## Abbreviations

acRoots: actinidia Chinensis Planch roots
AR: amphiregulin
CYP: cytochrome P450 monooxygenases
DDR: discoidin domain receptor
EGFR: epidermal growth factor receptor
GPR12: G protein-coupled receptor 12
HSPA6: heat shock protein family A member 6
IL21R: interleukin 21 receptor
LPS: lipopolysaccharide
NUPR1: nuclear protein 1
POTED: putrescine/proton symporter ankyrin domain family member D
RARB: retinoic acid receptor beta
siRNA: small interfering RNA
TGF-alpha: transforming growth factor-alpha

## Competing interests

The authors declare no competing of interests.

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## Authors' contributions

Conceived and designed the study: Lin Shi; Performed the biological experiments: Lingyan Wang, Jiayun Hou and Minghuan Zheng; Statistical analysis: Jiayun Hou and Minghuan Zheng. Wrote the paper: Lingyan Wang and Lin Shi. All authors read and proofed the final manuscript.

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