Original Article

Tumor Biology

Tumor Biology
June 2017: 1–13

DOI: 10.1177/1010428317705334
journals.sagepub.com/home/tub
SAGE

Identification of curcumin-inhibited extracellular matrix receptors in non–small cell lung cancer A549 cells by RNA sequencing

Huiping Li[1,2], Hongjin Wu[3], Hongfang Zhang[3], Ying Li[3], Shuang Li[3], Qiang Hou[3], Shixiu Wu[3] and Shuan-Ying Yang[1]

Abstract

Curcumin is a potent anti-cancer drug in several types of human cancers. Despite of several preclinical and clinical studies of curcumin, the precise mechanism of curcumin in cancer prevention has remained unclear. In our study, we for the first time investigated whole transcriptome alteration in A549 non–small cell lung cancer (NSCLC) cell lines after treatment with curcumin using RNA sequencing. We found that lots of genes and signaling pathways were significantly altered after curcumin treatment in A549 cells. With bioinformatics approaches (gene ontology, Kyoto Encyclopedia of Genes and Genomes, and STRING), we found that those curcumin altered genes were not only the genes that induce cell death but also those extracellular matrix receptors and mitogen-activated protein kinase signaling pathway genes which regulate cell migration and proliferation. Among those significantly altered genes, eight genes (*COL1A1, COL4A1, COL5A1, LAMA5, ITGA3, ITGA2B, DDIT3*, and *DUSP1*) were further examined by quantitative reverse transcription polymerase chain reaction and western blot analysis in four non–small cell lung cancer cell lines. Both in cell lines and in mouse model, the extracellular matrix receptors including the integrin (*ITGA3* and *ITGA2B*), collagen (*COL5A1*), and laminin (*LAMA5*) were significantly inhibited by curcumin at messenger RNA and protein levels. Functional studies confirmed that curcumin not only induced A549 cell death but also repressed cell proliferation and migration by regulating extracellular matrix receptors. Collectively, our study suggests that curcumin may be used as a promising drug candidate for intervening lung cancer in future studies.

Keywords

Curcumin, RNA sequencing, lung cancer, cell migration, extracellular matrix receptors

Date received: 18 October 2016; accepted: 11 January 2017

Introduction

Curcumin is the main bioactive component of Indian spice turmeric which is widely used in Indian and Chinese medicines for centuries.[1] Curcumin possesses various therapeutic roles including anti-cancer, anti-microbial, anti-oxidant, and anti-inflammatory activities.[1] Curcumin can attenuate cancer cell proliferation and induce cell apoptosis by regulating multiple signaling pathways such as p53, Ras, PI3K, AKT, Wnt/β-catenin, and mTOR (mechanistic target of rapamycin).[2] Curcumin can also inhibit cancer cell migration, invasion, and metastasis by altering the expression of adhesion molecules, inflammatory factors, growth factors,

[1]Department of Respiratory Medicine, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China
[2]Department of Emergency Medicine, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China
[3]Cancer Research Institute, Hangzhou Cancer Hospital, Hangzhou, China

Corresponding authors:
Shuan-Ying Yang, Department of Respiratory Medicine, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shanxi 710004, China.
Email: yangshuanying66@163.com

Shixiu Wu, Cancer Research Institute, Hangzhou Cancer Hospital, Hangzhou, Zhejiang 310002, China.
Email: wushixiu@medmail.com.cn

and growth factor receptors.[3–7] Some clinical trials confirm that curcumin is a potent anti-cancer drug with safety and minimal toxicity in patients.[8]

Lung cancer is one of the deadliest cancers with high morbidity and mortality in the world.[9] Although new chemotherapeutic drugs and treatment approaches have been developed in lung cancer treatment, the prognosis of patients remains poor.[10] During the past decades, several studies demonstrated that curcumin, as a naturally occurring polyphenol compound, showed promising in vitro and in vivo anti-cancer activity in lung cancer.[11] Curcumin suppressed lung cancer cell growth and induced cell death through inducing autophagy and inhibiting Bcl-2, cyclin D1, cyclin-dependent kinase 2 (CDK2), and CDK4.[12,13] By downregulating epidermal growth factor receptor (EGFR) expression, curcumin can block EGFR downstream tyrosine kinase signaling cascade and overcome acquired EGFR–tyrosine kinase inhibitors (TKIs) resistance.[14–16] By inhibiting Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling pathway, curcumin attenuated the stem-like traits of lung cancer cells and enhanced the anti-tumor effects of cisplatin.[17,18] But, until now, there have never been studies investigating the effects of curcumin on lung cancer cells at whole transcriptome level. Herein, we used RNA sequencing (RNA-seq; a well-developed tool to analyze the eukaryotic transcriptome) to gain a global view of curcumin-induced whole transcriptome alteration in human non–small cell lung cancer (NSCLC) cell line A549. After a systemic analysis of curcumin-induced whole transcriptome alteration, our study provided precise molecular targets and signaling pathways that curcumin regulated, which contributes to better application of curcumin in lung cancer treatment.

Materials and methods

Reagents and cell culture

Antibody against ITGA3 was purchased from Millipore (AB1920; Billerica, MA, USA). Antibody against LAMA5 was purchased from Biorbyt (orb214177; San Francisco, CA, USA). COL5A1 antibody was obtained from Santa Cruz Biotechnology (sc-133162; CA, USA). ITGA2B antibody was obtained from Abcam (ab134131; Cambridge, UK). Ki67 (#9449) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; #2118) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Protein concentration was determined with bicinchoninic acid (BCA) kit that was purchased from Thermo Fisher scientific (Waltham, MA, USA). Curcumin was obtained from Selleck Chemicals (S1848; Houston, TX, USA) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) to make stock solution.

Human NSCLC cell lines A549, H1299, H460, and PC9 were obtained from American Type Culture Collection and cultured in RPMI-1640 medium (Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (FBS) at 37°C in 5% CO_2/95% air.

RNA-seq library preparation and sequencing

Total RNA was isolated from 4×10^6 A549 cells treated with or without curcumin using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The integrity of total RNA was assessed using an Agilent 2100 Bioanalyzer (Life Technologies, Carlsbad, CA, USA) with a RNA integrity number (RIN) greater than 8. After two rounds of purification using poly-T oligo-attached magnetic beads, the messenger RNA (mRNA) was purified from total RNA (2 μg) and fragmented. Cleaved RNA fragments were primed with random hexamers and then reverse transcribed into first-strand complementary DNA (cDNA) using reverse transcriptase and random primers. The RNA template was removed and a replacement strand was synthesized to generate double-stranded cDNA. End repair, A-tailing, adaptor ligation, cDNA template purification, and enrichment of the purified cDNA templates were then performed using polymerase chain reaction (PCR). Constructed libraries were 100 bp paired-ends and sequenced by Illumina Hiseq2000 sequencer (Suzhou Zhongxin Biotechnology Co., Ltd, Suzhou, China). The RNA-seq data in our study had been submitted to Sequence Read Archive (SRA) database, with SRA number SRP065304.

RNA sequencing data alignment

We aligned the sequencing reads to a human genome reference (hg19) using TopHat version 2.0.13. Splice junctions were extracted from RefSeq alignment (downloaded from the University of California, Santa Cruz (UCSC) Genome Browser). The resulting read alignments were assembled through Cufflinks version 1.1, and it created a novel transcript using Cufflinks reference annotation-based transcript (RABT) algorithm. The transcripts combined with Cuffcompare were used to calculate relative abundances of each transcript through Cuffdiff. Gene expression levels were determined by measuring the sum of fragments per kilobase of exon model per million mapped reads (FPKM) values of its exons. The RNA-seq data were imported into R for Volcano plot using statistical test (two-factor analysis of variance (ANOVA)) with an adjusted p value of 0.05 for significance.

RNA extraction and real-time PCR

Total RNA was extracted from cancer cells using the TRIzol Reagent (Invitrogen, Life technologies, Carlsbad, CA, USA) following the manufacturer's instructions.

Reverse transcription was performed with Fermentas K1622 (Thermo Fisher scientific, Waltham, MA, USA) following the manufacturer's instructions. Quantitative reverse transcription PCR (qRT-PCR) was conducted using SYBR Green (ABgene, Epsom, UK) according to the manufacturer's instructions. Primer sequences were listed in Supplementary Table S1.

Cell Counting Kit-8 assay

The cell viability after curcumin treatment was determined with Cell Counting Kit-8 (CCK8) assay. Briefly, 3000 cells per well were seeded onto 96-well plate and incubated overnight. Then, the cells were treated with different concentrations of curcumin. After incubation for 48 h, the medium in each well was replaced with fresh culture medium containing 10 μL CCK8 (Dojindo Molecular Technologies, Kumamoto, Japan). The plates were incubated for additional 3 h, and then, the absorbance was determined at 450 nm with a microplate spectrophotometer (SpectraMAX M2e; Molecular Devices, Silicon Valley, CA, USA).

Colony formation assay

Cells were seeded onto six-well plates. After adherent growth, the cells were treated with 8 μg/mL curcumin or 0.1% DMSO as a control and incubated for 9 days. After washing with phosphate-buffered saline (PBS), the cells were fixed with methanol for 10 min at room temperature, and then, the crystal violet solution (C0121; Beyotime, Shanghai, China) was added onto the plates and incubated for 30 min at 37°C. Then, the plates were washed with deionized water and dried at room temperature. The colonies in each plate were counted by two experienced researchers.

Wound healing assay

Cells were seeded onto six-well plates and cultured to adherent growth. The adherent monolayer cells were scratched with a sterile 10 μL pipette tip to create a narrow wound-like gap. After washing twice with sterile PBS to remove loose cells and cell debris, the medium was replaced with fresh FBS-free medium containing 8 μg/mL curcumin or 0.1% DMSO (v/v) as a control. After 48 h, the wounds were photographed under the microscope.

Flow cytometry analysis

After treatment with 8 μg/mL curcumin for 24 or 48 h, A549 cells were harvested, washed with ice-cold PBS, and re-suspended in binding buffer. Cells treated with 0.1% DMSO (v/v) were used as a control. Cell suspension was stained with Alexa Fluor 488–conjugated annexin V (AV) and propidium iodide (PI) at room temperature in the dark. After incubation for 5 min, cells were analyzed by FC500 flow cytometer (Beckman–Coulter, CA, USA). The CXP CellQuest software (Beckman–Coulter) was used to count the number of apoptotic (Alexa Fluor 488+/PI− and Alexa Fluor 488+/PI+) and necrotic (Alexa Fluor 488−/PI+) cells.

Western blot analysis

Cells were harvested after indicated treatments and washed twice with ice-cold PBS before adding into protein extraction buffer. Equal amount of proteins was fractionated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% non-fat milk, PVDF membrane was incubated with diluted primary antibodies (GAPDH: 1:5000 dilution, Ki67: 1:5000 dilution, LAMA5: 1:1000 dilution, COL5A1: 1:1000 dilution, ITGA2B: 1:1000 dilution, and ITGA3: 1:1000 dilution) at 4°C overnight. Then, the membrane was washed with Tris buffered saline with Tween 20 (TBST) three times at room temperature and incubated with corresponding horseradish peroxidase (HRP)-linked secondary antibody for 2 h at room temperature. Proteins were ultimately visualized by enhanced chemiluminescence and autoradiography (Thermo Fisher Scientific, Waltham, MA, USA).

Xenograft transplantation and therapy

Four-week-old BALB/c nude mice were purchased from SLAC laboratory Animal Co., Ltd (Shanghai, China) and maintained under standard conditions in Experimental Animal Center in Zhejiang Chinese Medicine University (Zhejiang, China). All the animal protocols in our study were in accordance with the institutional animal welfare guidelines of Zhejiang Chinese Medicine University. To develop xenograft tumors, in vitro growing A549 cells were harvested by exposing to trypsin–ethylenediaminetetraacetic acid (EDTA) and washed with ice-cold PBS. A volume of 5×10^6 A549 cells in 100 μL PBS were implanted into the right flanks of BALB/c nude mice. Two weeks later, tumor-bearing mice were intraperitoneally administrated with 0.1% DMSO or 150 mg/kg curcumin daily for three consecutive days. After 10 days, all mice were sacrificed according to institutional guidelines, and tumors were resected for protein extraction. Tumor volume (mm^3) was calculated using the following formula: $V\ (mm^3) = A\ (mm) \times B\ (mm^2) / 2$, where A and B were the longest and widest diameter of tumor, respectively, and measured for every 2 days by a caliper.

Database for Annotation, Visualization and Integrated Discovery analysis

Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov), Functional Annotation Bioinformatics Microarray Analysis

was used to identify significantly enriched gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms among the given list of genes that are differentially expressed in response to curcumin. Statistically overrepresented GO and KEGG categories with $p \leq 0.01$ were considered significant.

Statistical analysis

Statistical data were represented as mean ± standard deviation from at least three independent experiments performed in triplicate. The colony formation, gene expression, and tumor volume were statistically analyzed using repeated measures two-way ANOVA.

Results

Identification of curcumin-induced transcriptome alteration in A549 cells through RNA-seq analysis

In order to determine experimental conditions for transcriptome analysis, we performed the cytotoxicity assay of curcumin with A549 cancer cells. As shown in Supplementary Figure S1, curcumin inhibited the viability of A549 cells in a dose-dependent manner. Among the tested doses, we found that the dosage of 8 μg/mL curcumin which induced 44.7% cell viability was the closest to the half maximal inhibitory concentration (IC_{50}) of curcumin after incubation for 48 h in A549 cells (Supplementary Figure S1). So, the transcriptome analysis was performed on A549 cells after treatment with 8 μg/mL curcumin for 48 h. The schematic diagram of our study was presented in Figure 1(a). We acquired valid RNA-seq data from curcumin-treated (2,728,030 bp) and non-treated (2,902,959 bp) A549 cells with Q30 > 90%, respectively (Supplementary Table S2). We filtered out our data with estimated FPKM values less than 1.0 in samples for more accurate results. Ultimately, with statistical analysis (Volcano plot), we found that the transcriptome of curcumin-treated A549 cells were significantly different from those of untreated cells (Figure 1(b)). Totally 1993 differentially expressed genes (DEG) were identified in curcumin-treated A549 cells compared with untreated A549 cells. Among these genes, 1028 genes were upregulated at least two-fold and 965 genes were downregulated at least two-fold (Supplementary Table S3). The heatmap for some significantly altered genes by curcumin was shown in Figure 1(c). According to cellular functional categorization, significantly upregulated genes were classified as follows ($p \leq 0.01$): Steroid metabolic process (*CYP3A5, AKR1C4, CYP1A1, INSIG2, SULT1A1, PCSK9, NR5A2, SCP2, AKR1C1*, and *DHCR24*), negative regulation of cell proliferation (*HYAL1, CTTNBP2, CDC6, RBP4, CTH, ANG, GDF11, AXIN2, GPNMB, FOXO4, BMP7*, and *DHCR24*), cell motion (*PLAT, VAV3, S100P, KIF5C, PF4, SCYL3, ITGAM, EPHA4, CTTNBP2, DOCK2, SAA2, ANK3, ANG*, and *BMP7*), and cell migration (*PLAT, CTTNBP2, DOCK2, S100P, VAV3, SAA2, ANG, PF4, SCYL3*, and *ITGAM*). The significantly downregulated genes were classified as follows ($p \leq 0.01$): response to virus (*IFIH1, ZC3HAV1, RSAD2, CCL5, APOBEC3F, IFI35, ISG20, ISG15, CXCR4, BCL2, MX1, MX2, FOSL1, IL6, BST2, IFI44, IFI16, STAT1, TRIM22, DDX58, IRF9, PLSCR1, IFNB1, LYST*, and *IRF7*), positive regulation of developmental process (*TNFRSF12A, CSF1, FST, BTC, TGFB3, OXTR, TNFSF12, CCL5, GLI2, CDH4, SHH, EPHB2, TGFB2, LIF, METRN, BDNF, CYP27B1, GATA6, BCL2, HLX, GATA4, JUND, RHOB, RARA, IL2RG, HSF4, RUNX2, INSR, ARHGDIA, IL6, TESC, SOCS3, SPHK1, SMAD1, IL6R, NTRK3, CD83, SERPINF1, ETS1, JUN, JAK2, BMPR1B*, and *IGFBP3*), and regulation of cell migration (*PDGFB, CSF1, EDN1, TPM1, SHH, CITED2, TGFB2, CXCL10, NISCH, CXCR4, BCL2, INSR, COL18A1, ICAM1, IL6, IRS2, PTPRM, EGFL7, SPHK1, SCAI, IL6R, IRS1, LAMA5, CXCL16, PTP4A1, JAK2, MUC5AC*, and *IGFBP3*) (Supplementary Table S3).

Identification of curcumin regulated signaling pathways in A549 cells through DAVID and STRING analysis

After bioinformatics analysis of RNA-seq data, the upregulated or downregulated genes in curcumin-treated A549 cells were summarized. Furthermore, we submitted these genes to DAVID (a web-based high-throughput functional genomics analysis tool) for systematically clustering these genes. In GOTERM_BP_FAT category, we identified significantly enriched GO terms and characterized curcumin-induced responses of A549 cells. In total, 4 subcategories ($p \leq 0.01$) were found among upregulated genes and 66 subcategories ($p \leq 0.01$) among downregulated genes (Supplementary Table S4). In KEGG_PATHWAY category, the upregulated genes were clustered into only 2 subcategories ($p \leq 0.05$) and downregulated genes were clustered into 11 subcategories ($p \leq 0.05$, 6 subcategories with $p \leq 0.01$) (Supplementary Table S5).

Based on transcriptome analysis, we found that curcumin mainly induced downregulation but not upregulation of target genes in A549 cells. The following signaling pathways were significantly altered in A549 cells after treatment with curcumin: small cell lung cancer, pathways in cancer, systemic lupus erythematosus, mitogen-activated protein kinase (MAPK) signaling pathway, extracellular matrix (ECM)-receptor interaction, and cytokine-cytokine-receptor interaction (Figure 1(d)).

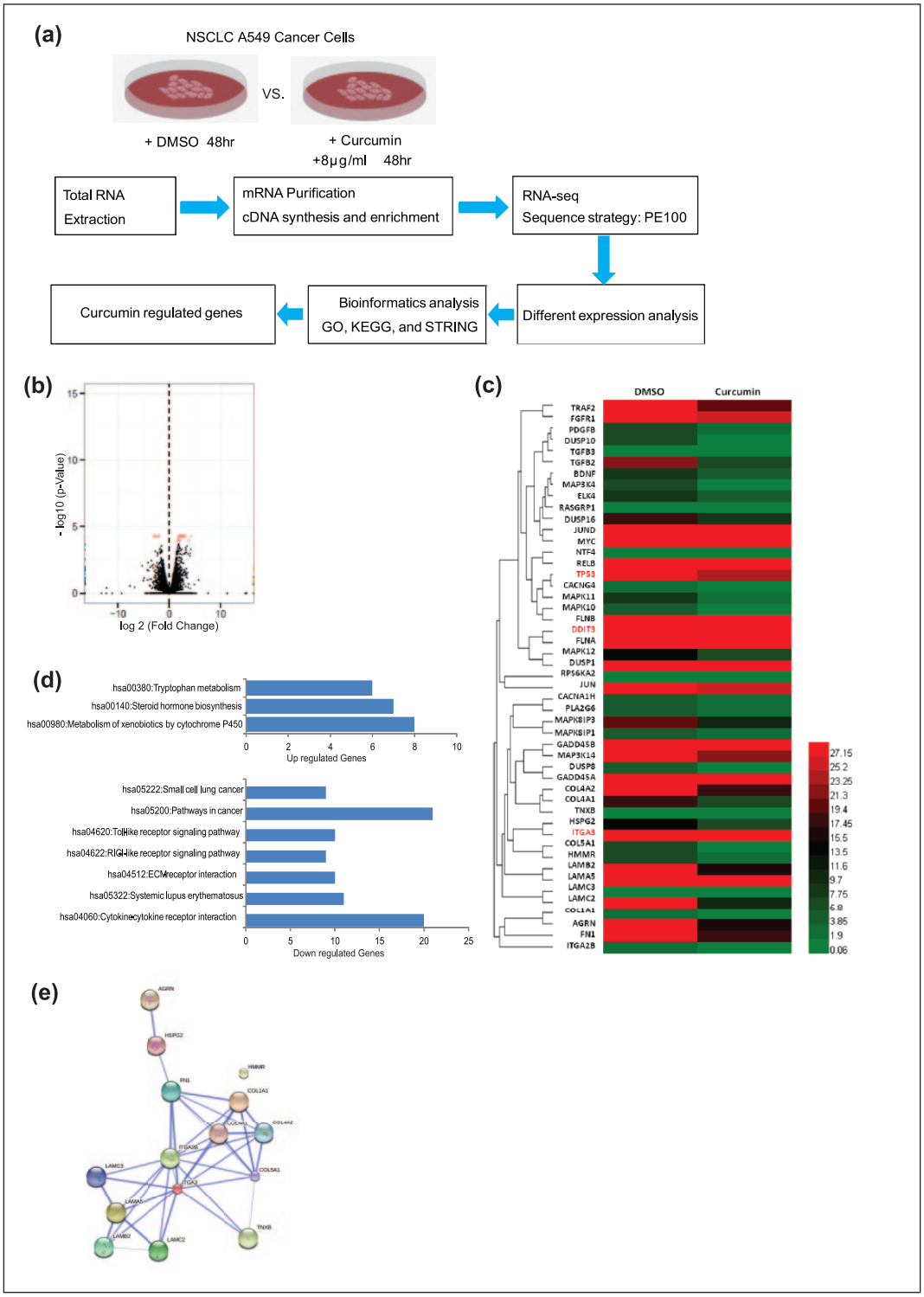

Figure 1. Transcriptome alteration analysis with RNA-seq in A549 NSCLC cell lines after curcumin treatment. (a) A schematic diagram of the design and goals of our study. (b) Volcano plot analysis of transcriptome alteration in A549 cells with or without curcumin treatment. (c) Heatmap analysis of the altered genes in curcumin-treated A549 cells. Red: high expression level; green: low expression level. (d) KEGG pathway analysis of the altered genes in curcumin-treated A549 cells by DAVID. (e) STRING analysis of the altered ECM-receptor genes in curcumin-treated A549 cells.

These results with bioinformatics analysis were matched with cellular functions of curcumin in A549 cells that include the induction of cell death and the repression of cell proliferation (signaling pathways: small cell

lung cancer, pathways in cancer, and MAPK signaling pathway). The ECM-receptor interaction pathway was repressed after curcumin treatment, suggesting that curcumin may also repress A549 cell migration. Then, we performed STRING analysis of all downregulated ECM-receptor genes and found that *COL1A1, COL4A1, COL5A1, LAMA5, ITGA3, ITGA2B*, and *DDIT3* were in the same protein–protein interaction network, suggesting that curcumin may repress cancer cell migration through downregulation of massive ECM-receptor genes (Figure 1(e)).

Real-time RT-PCR validation of RNA-seq results

Through GO and KEGG analysis, we found that curcumin may not only induce cancer cell death but also affect cancer cell migration and proliferation through downregulating ECM receptors and MAPK-related genes. To validate the accuracy of RNA-seq results, 10 potential target genes of curcumin (*COL1A1, COL4A1, COL5A1, LAMA5, ITGA3, ITGA2B, DDIT3, DUSP1, JUN*, and *MAPK11*) were selected and analyzed by qRT-PCR. We found that the changes in these genes at mRNA levels by qRT-PCR were consistent with the RNA-seq results (Figure 2(a) and (b)). In addition, these results suggested that our RNA-seq data were highly reliable and can be used to investigate the biological effects of curcumin on NSCLC cell lines.

Cellular functions of curcumin in A549 cancer cells

Since the RNA-seq analysis revealed that curcumin may not only cause cell death but also repress cell migration and proliferation. We analyzed curcumin-induced cell death by fluorescence-activated cell sorting (FACS) analysis (Figure 3(a)), cell proliferation by colony formation assay and western blot analysis (Figure 3(b) and (c)), and cell migration by wound healing assay (Figure 3(d)). Compared with DMSO-treated group, curcumin caused massive A549 cell death (24.1% plus 6.3%) at a concentration of 8 μg/mL for 24 h which further increased (48.2% plus 1.5%) after 48 h incubation (Figure 3(a)). Furthermore, curcumin significantly repressed colony formation of A549 cells (Figure 3(b)) and the protein level of a proliferation marker Ki67 (Figure 3(c)). Wound healing assay was performed to investigate the inhibitory effects of curcumin on A549 cell migration. After 48 h treatment, we found that compared with DMSO-treated A549 cells, the migration of curcumin-treated A549 cells were significantly repressed (Figure 3(d)). Based on the RNA-seq analysis, we suggest that the downregulation of ECM receptors by curcumin may play a critical role in the repression of A549 cell death, proliferation, and migration.

ECM receptors were significantly downregulated by curcumin in vitro and in vivo

When treated with curcumin at a concentration of 8 μg/mL (A549, H1299, and H460) or 4 μg/mL (PC9), the expression of ECM receptors was significantly repressed (Figure 4(a)–(d)). Furthermore, western blot analysis was performed on the extractions of A549, H1299, H460, and PC9 cells treated with or without 8 μg/mL or 4 μg/mL curcumin for 48 h. Consistent with the RNA-seq and qRT-PCR results, the ECM receptors including *ITGA3, COL5A1*, and *LAMA5* were obviously inhibited at protein level after treatment with curcumin (Figure 4(e)). In xenograft tumors, curcumin significantly reduced tumor size (Figure 5(a) and (b)). In addition, the expression levels of ECM receptors (*ITGA3, ITGA2B, COL5A1*, and *LAMA5*) were detected in tumor samples with or without curcumin treatment. As shown in Figure 5(c), we found that the expression of these proteins was repressed in all curcumin-treated tumors. Therefore, our study suggests that downregulation of ECM receptors by curcumin may be one of the major causal factors for A549 cell death and the repression of cell proliferation and migration.

ECM receptors were highly expressed in human cancers with ONCOMINE analysis

Since the expression of ECM receptors was significantly inhibited by curcumin, our study investigated whether ECM receptors were highly expressed in human cancer tissues. The expression levels of ECM receptors, including integrin (*ITGA3* and *ITGA2B*), collagen (*COL1A1, COL4A1*, and *COL5A1*), and laminin (*LAMA5*), were examined by ONCOMINE. Compared with human normal tissues, ECM receptors except for *ITGA2B* were significantly highly expressed in human cancer tissues (Figure 6(a)). In lung cancer, we found that the collagens (*COL1A1* and *COL5A1*) were significantly upregulated either in lung adenocarcinoma or in squamous cell lung carcinoma (Figure 6(b)). Therefore, curcumin, as a potent inhibitor of ECM receptors, can be used for treating human cancers with highly expressed ECM receptors.

Discussion

This study investigated curcumin-regulated molecular targets and signaling pathways at whole transcriptome level by RNA-seq in A549 NSCLC cell lines. Many signaling pathways were altered in curcumin-treated A549 cells, including cell apoptosis. This is consistent with previous studies which showed that curcumin induces cancer cell apoptosis via multiple targets such as Notch signaling, nuclear factor kappa B (NF-κB) pathway, and PI3K/AKT signaling pathway.[19–25] Our RNA-seq data reveals

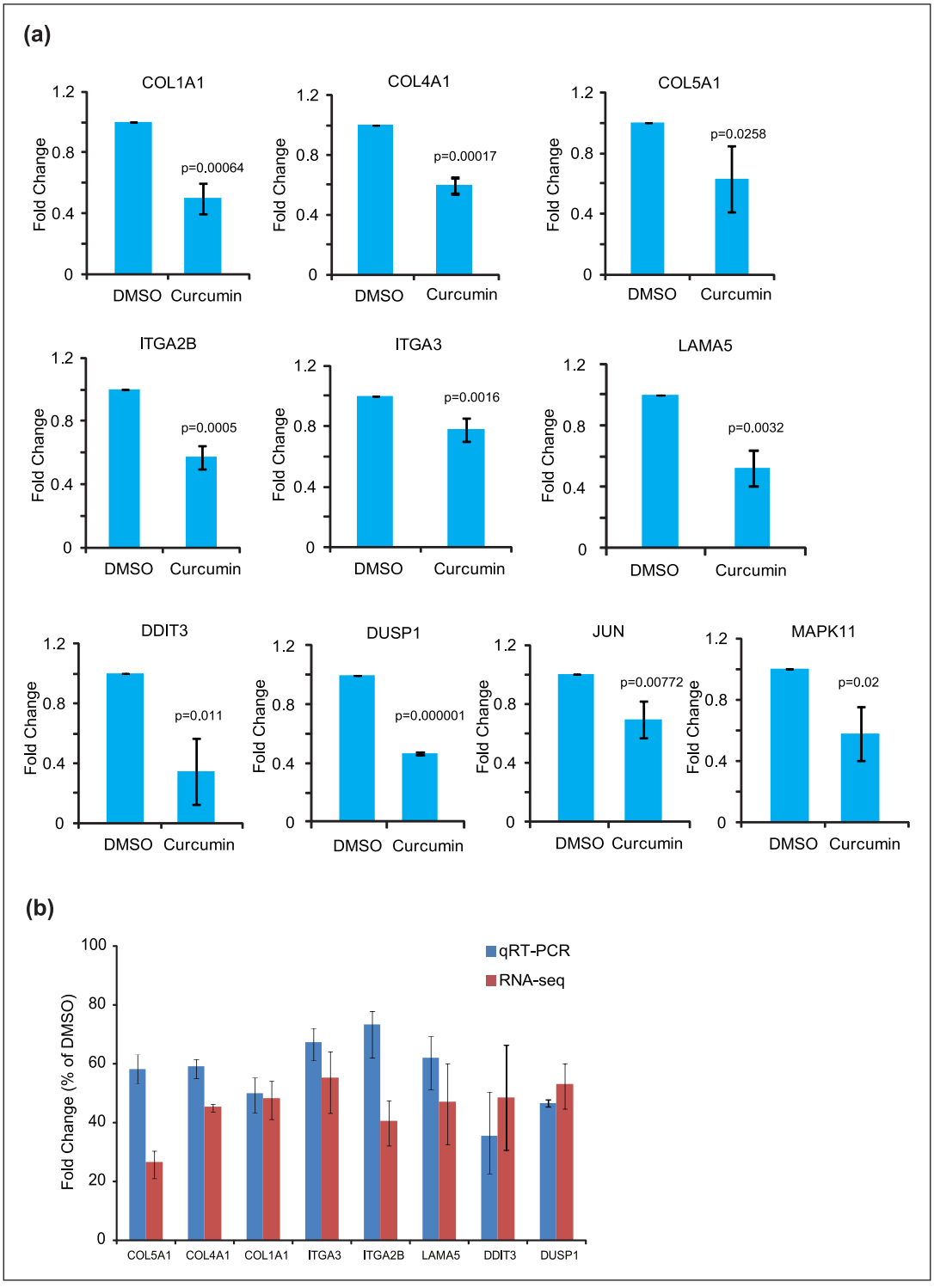

Figure 2. Validation of our RNA-seq data with qRT-PCR. (a) The expression of target genes was analyzed by qRT-PCR. (b) The comparison of gene expression was determined with RNA-seq and qRT-PCR in curcumin-treated group compared with in DMSO-treated group.

that curcumin has significant inhibitory effects on the expression of ECM receptors, including collagen (*COL1A1, COL4A1*, and *COL5A1*), integrin (*ITGA2B* and *ITGA3*), and laminin (*LAMA5*) in A549 cells. This result was also confirmed in other NSCLC cell lines and xenograft tumor models. Curcumin downregulates the

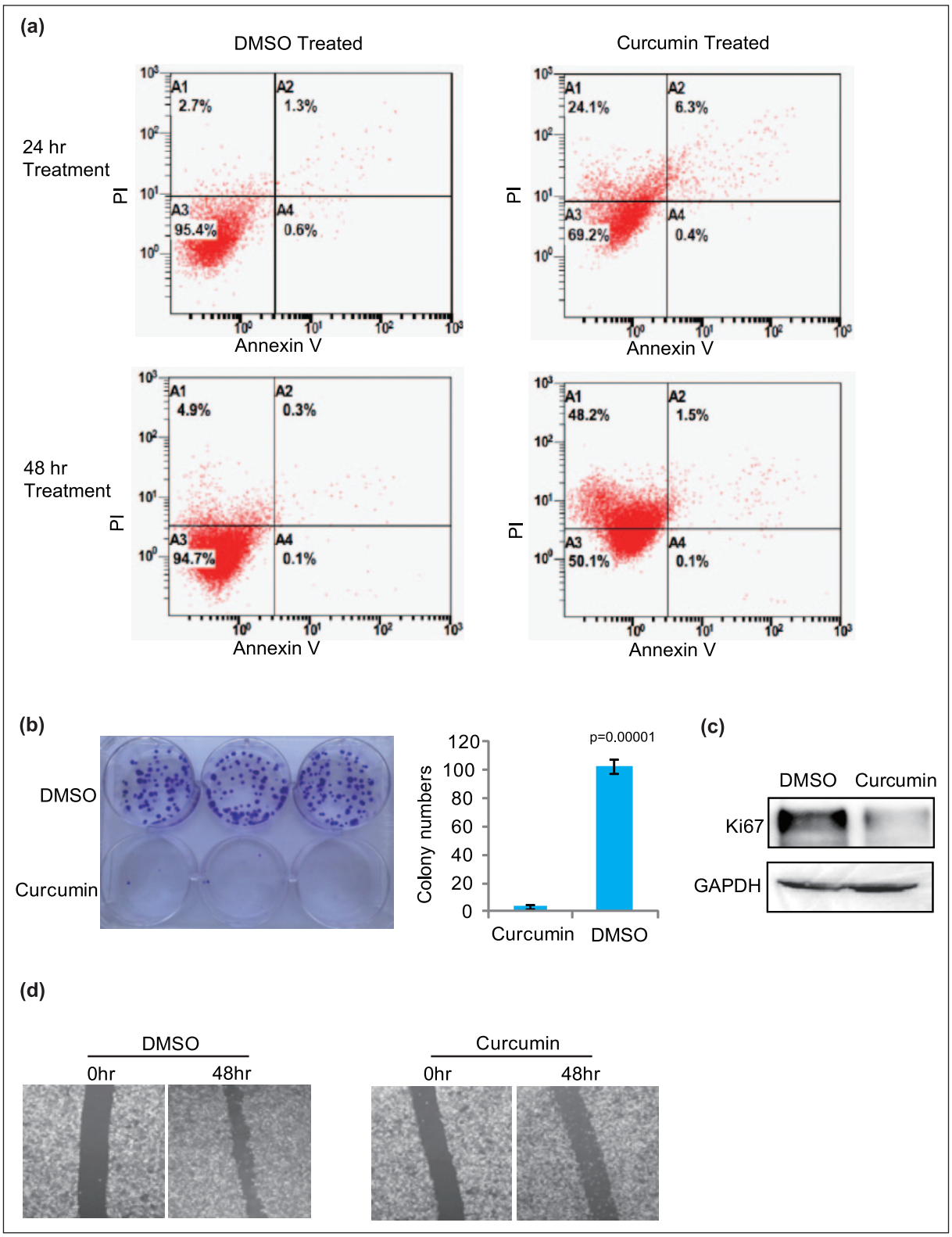

Figure 3. Curcumin induced cell death and repressed cell migration in A549 NSCLC cell lines. (a) FACS analysis of curcumin-induced A549 cell death. A549 cells treated with or without 8 μg/mL curcumin were stained with Annexin V–Alex Fluor 488/PI Apoptosis Detection Kit and analyzed by FACS. After curcumin treatment, massive A549 cells underwent necrosis from 24.1% (24 h treatment) to 48.2% (48 h treatment). (b) A549 cell growth inhibition by curcumin was analyzed by clone formation assay. A549 cells were placed onto the plate, with or without 8 μg/mL curcumin treatment. After 9 days, the clones were dyed with crystal violet and counted. (c) Western blot analysis of the expression of Ki67 in A549 cells treated with or without 8 μg/mL curcumin. GAPDH was used as a loading control. (d) The inhibition of A549 cell migration by 8 μg/mL curcumin for 48 h was analyzed by wound healing assay.

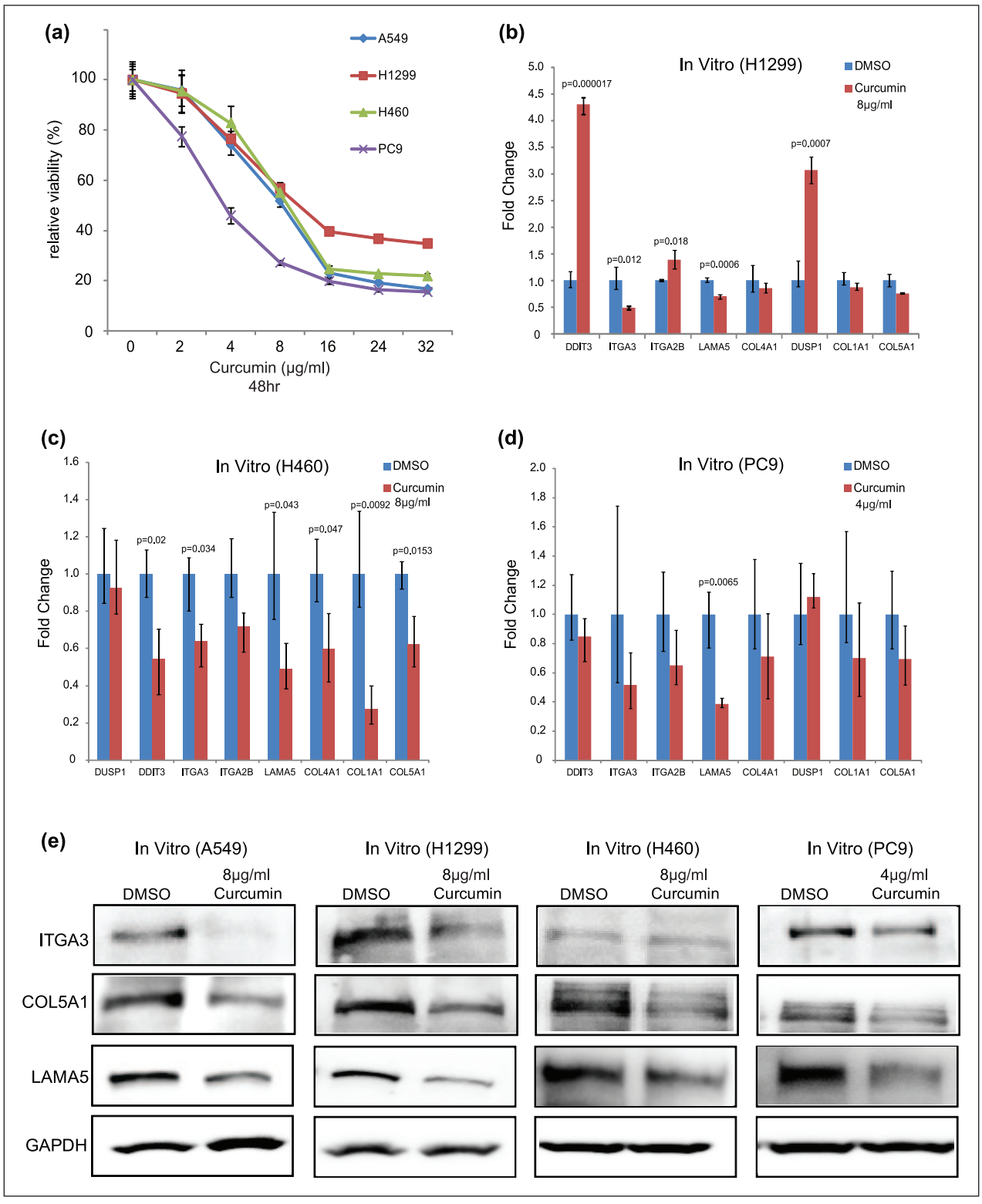

Figure 4. Curcumin inhibited the expression of ECM receptors in various human NSCLC cell lines. (a) The viability of human NSCLC cell lines (H1299, H460, PC9, and A549) after treatment with different concentrations of curcumin for 48 h was analyzed by CCK8 assay. The mRNA levels of ECM receptors including *DDIT3*, *ITGA3*, *ITGA2B*, *LAMA5*, *COL4A1*, *DUSP1*, *COL1A1*, and *COL5A1* in (b) H1299, (c) H460 and, (d) PC9 after treatment with 8 μg/mL or 4 μg/mL curcumin for 48 h were quantified by qRT-PCR. (e) The protein expression of ITGA3, COL5A1, and LAMA5 in H1299, H460, PC9, and A549 after treatment with 8 μg/mL or 4 μg/mL curcumin for 48 h was analyzed by western blot analysis. GAPDH was used as a loading control.

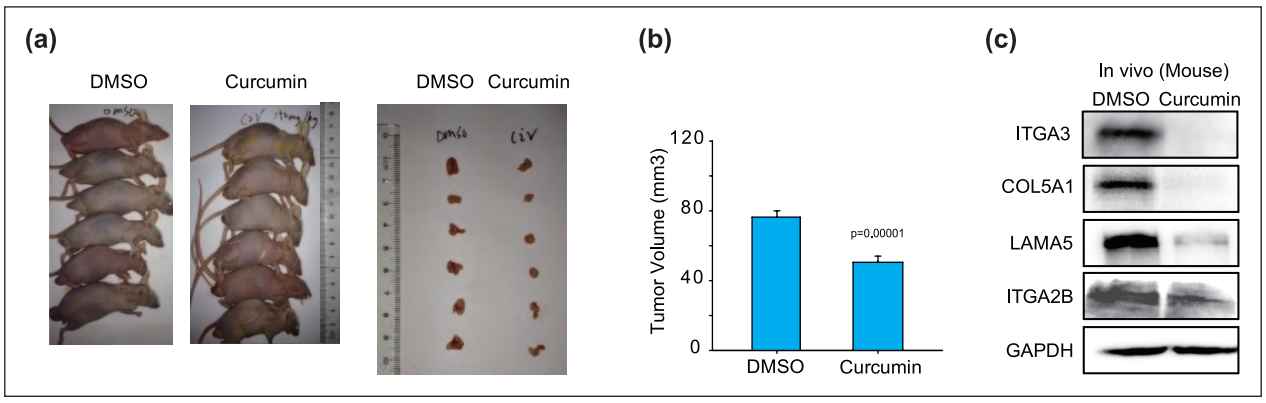

Figure 5. ECM receptors were significantly inhibited by curcumin in xenograft tumors. (a) The photos of tumor-bearing mice and excised xenograft tumors with or without curcumin treatment (150 mg/kg curcumin daily for three consecutive days) at the end of the experiment were shown. (b) The volumes of xenograft tumors with or without curcumin treatment (150 mg/kg curcumin daily for three consecutive days) at the end of the experiment. (c) Western blot analysis of the expression of ECM receptors in excised xenograft tumors with or without curcumin treatment (150 mg/kg curcumin daily for three consecutive days). GAPDH was used as a loading control.

expression of ECM receptors at both the RNA and protein levels. So we suggest that by regulating the ECM-receptor interaction pathway, curcumin inhibited cell proliferation and invasion and induced death in A549 cancer cells.

The ECM receptors are transmembrane proteins that are required for cell–cell binding or cell binding to extracellular molecules.[26] The aberrant activation of the ECM-receptor interaction pathway is involved in cell survival, proliferation, migration, polarity, and differentiation.[27–29] The adhesion of cancer cells to ECM via collagen and integrin induced the activation of various pro-survival pathways such as PI3K/Akt, p53/MAPK, extracellular signal–regulated kinase (ERK)/MAPK, and Rho/ROCK conferring resistance to chemotherapeutic agents.[30–33] In vivo studies show that high expression of ECM receptors is closely associated with a poor prognosis of patients through increasing chemoresistance and metastatic potential.[34] Collagen XVII and laminin-5 are linked to the activation of epithelial–mesenchymal transition (EMT) and poor prognosis in lung cancer patients.[35] In NSCLC, integrin regulated cancer cell invasion, metastasis, and chemoresistance.[36–40] Integrin inhibitors have been developed to reverse malignant phenotype of human cancers. Some of the inhibitors have entered into clinical trials, which can significantly improve cancer patients' survival rate.[41,42] Volociximab, an α5β1 inhibiting antibody, in combination with carboplatin and paclitaxel, has completed a phase II study, for advanced NSCLC.[43] Kumar et al.[44] found that curcumin inhibited tumor necrosis factor (TNF)-mediated adhesion of monocytes to endothelial cells by suppressing cell surface expression of adhesion molecules and of nuclear factor-κB activation. Herein, our findings show that ECM receptors including collagen, integrin, and laminin are the main therapeutic targets of curcumin, and curcumin can be used as another promising drug candidate to intervene in NSCLC.

In addition to lung cancer, our bioinformatics analysis revealed that ECM receptors including collagen (*COL1A1, COL4A1*, and *COL5A1*), integrin (*ITGA2B* and *ITGA3*), and laminin (*LAMA5*) are also highly expressed in many other human cancers, such as colorectal cancer, esophagus cancer, gastric cancer, and head and neck cancer. In these cancers, curcumin may also have potent anti-tumor activities by regulating the expression of ECM receptors. Our study discovered ECM receptors as the main targets of curcumin by RNA-seq in A549 cells, which suggests that curcumin may be used alone or in combination with other anti-cancer drugs to reverse ECM-receptors-associated malignant phenotype of human cancers. However, there are some limitations with our current study. Statistical analyses were not performed for some experiments including wound healing assay and cell death assays. The inhibitory effects of curcumin on lung cancer metastasis should be investigated in vivo by the establishment of a metastasis xenograft tumor model. To further ensure the efficacy of curcumin in lung cancer, more studies are needed including the assessment of safety, side effects, and pharmacokinetics of curcumin by in vivo studies. Furthermore, the precise mechanisms by which curcumin downregulates the expression of ECM receptors should be discovered.

Acknowledgements

The authors are indebted to all the individuals who consented to participate in this study. S.Y.Y. and S.X.W. conceived the study. H.P.L., H.J.W., Q.H., and Y.L. performed the experiments under the supervision of S.Y.Y. and S.X.W. H.J.W. analyzed the data. H.J.W. and H.F.Z. prepared the manuscript. S.X.W., S.Y.Y., H.P.L., and H.F.Z. reviewed the result and the discussion of the manuscript.

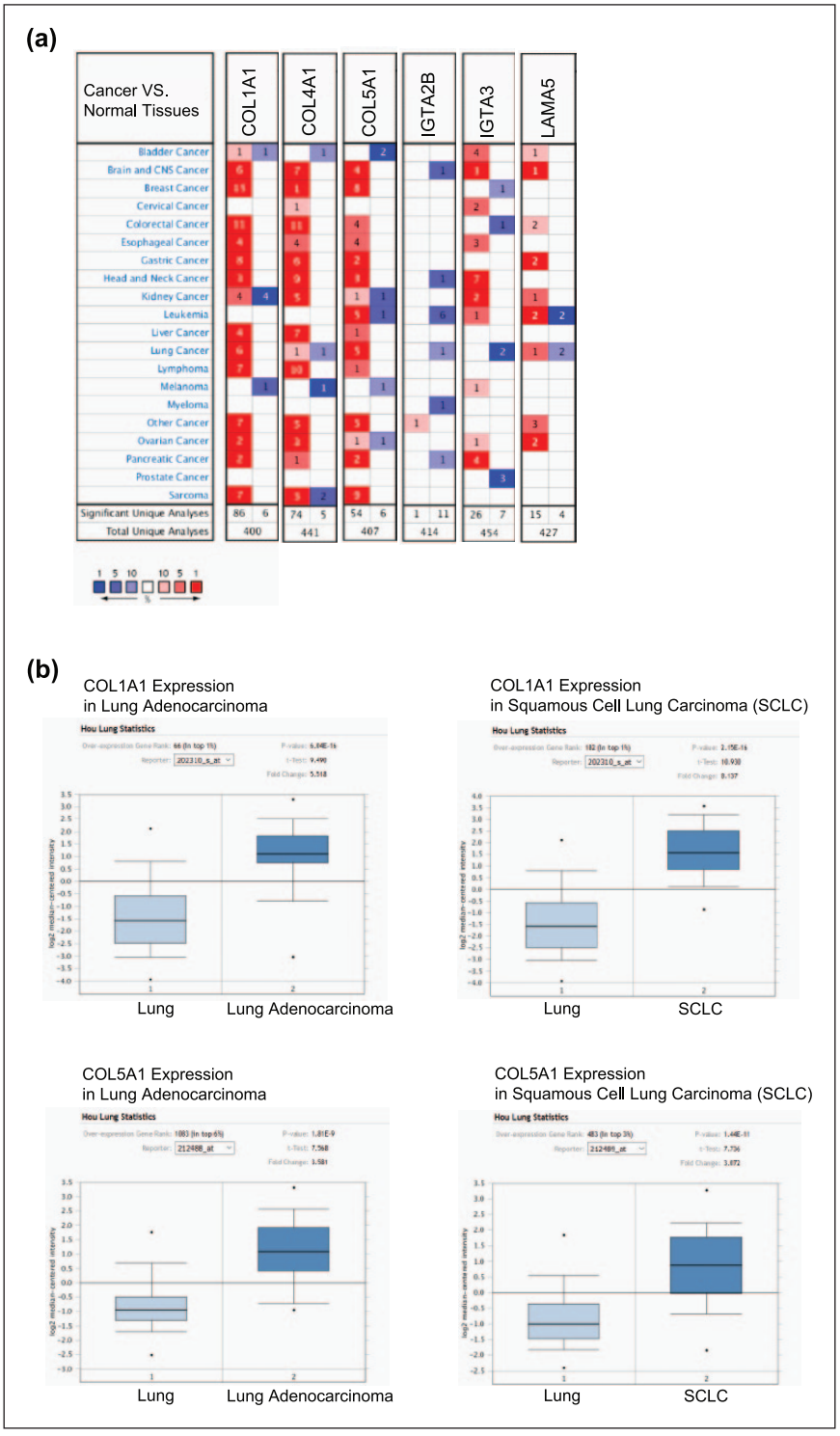

Figure 6. ECM receptors were significantly upregulated in various human cancers in ONCOMINE database. (a) The RNA expression of ECM receptors in various human cancer tissues and matched normal tissues in ONCOMINE database. Red: upregulated genes; blue: downregulated genes. (b) The RNA expression of COL1A1 and COL5A1 in lung adenocarcinoma and squamous cell lung carcinoma in ONCOMINE database.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was financially supported by Natural Science

Foundation of Zhejiang Province (LZ15H220001 and LY14H160005), Zhejiang medical and Health Ministry Training Program (2015PYA009), and National Natural Science Foundation of China (CN; 81402529).

References

1. Deng YI, Verron E and Rohanizadeh R. Molecular mechanisms of anti-metastatic activity of curcumin. *Anticancer Res* 2016; 36: 5639–5647.
2. Kasi PD, Tamilselvam R, Skalicka-Wozniak K, et al. Molecular targets of curcumin for cancer therapy: an updated review. *Tumour Biol* 2016; 37: 13017–13028.
3. Lee WH, Loo CY, Young PM, et al. Recent advances in curcumin nanoformulation for cancer therapy. *Expert Opin Drug Deliv* 2014; 11: 1183–1201.
4. Lin SS, Lai KC, Hsu SC, et al. Curcumin inhibits the migration and invasion of human A549 lung cancer cells through the inhibition of matrix metalloproteinase-2 and-9 and vascular endothelial growth factor (VEGF). *Cancer Lett* 2009; 285: 127–133.
5. Kim JM, Noh EM, Kwon KB, et al. Curcumin suppresses the TPA-induced invasion through inhibition of PKCα-dependent MMP-expression in MCF-7 human breast cancer cells. *Phytomedicine* 2012; 19: 1085–1092.
6. Wang SJ, Yu SJ, Shi W, et al. Curcumin inhibits the migration and invasion of mouse hepatoma Hca-F cells through down-regulating caveolin-1 expression and epidermal growth factor receptor signaling. *IUBMB Life* 2011; 63: 775–782.
7. Yang CL, Liu YY, Ma YG, et al. Curcumin blocks small cell lung cancer cells migration, invasion, angiogenesis, cell cycle and neoplasia through janus kinase-STAT3 signalling pathway. *PLoS ONE* 2012; 7: e37960.
8. Bimonte S, Barbieri A, Leongito M, et al. Curcumin anticancer studies in pancreatic cancer. *Nutrients* 2016; 8: E433.
9. Ramalingam SS, Owonikoko TK and Khuri FR. Lung cancer: new biological insights and recent therapeutic advances. *CA Cancer J Clin* 2011; 61: 91–112.
10. Goldstraw P, Crowley J, Chanksy K, et al. The IASLC Lung Cancer Staging Project: proposals for the revision of the TNM stage groupings in the forthcoming (seventh) edition of the TNM classification of malignant tumors (vol. 2, pp. 706–714, 2007). *J Thorac Oncol* 2007; 2: 985.
11. Mehta HJ, Patel V and Sadikot RT. Curcumin and lung cancer—a review. *Target Oncol* 2014; 9: 295–310.
12. Xiao K, Jiang JH, Guan CX, et al. Curcumin induces autophagy via activating the AMPK signaling pathway in lung adenocarcinoma cells. *J Pharmacol Sci* 2013; 123: 102–109.
13. Puliyappadamba VT, Cheriyan VT, Thulasidasan AKT, et al. Nicotine-induced survival signaling in lung cancer cells is dependent on their p53 status while its down-regulation by curcumin is independent. *Mol Cancer* 2010; 9: 220.
14. Li SQ, Liu ZL, Zhu F, et al. Curcumin lowers erlotinib resistance in non-small cell lung carcinoma cells with mutated EGF receptor. *Oncol Res* 2013; 21: 137–144.
15. Lee JY, Lee YM, Chang GC, et al. Curcumin induces EGFR degradation in lung adenocarcinoma and modulates p38 activation in intestine: the versatile adjuvant for gefitinib therapy. *PLoS ONE* 2011; 6: e23756
16. Lev-Ari S, Starr A, Vexler A, et al. Inhibition of pancreatic and lung adenocarcinoma cell survival by curcumin is associated with increased apoptosis, down-regulation of COX-2 and EGFR and inhibition of Erk1/2 activity. *Anticancer Res* 2006; 26: 4423–4430.
17. Wu LC, Guo LX, Liang YH, et al. Curcumin suppresses stem-like traits of lung cancer cells via inhibiting the JAK2/STAT3 signaling pathway. *Oncol Rep* 2015; 34: 3311–3317.
18. Baharuddin P, Satar N, Fakiruddin KS, et al. Curcumin improves the efficacy of cisplatin by targeting cancer stem-like cells through p21 and cyclin D1-mediated tumour cell inhibition in non-small cell lung cancer cell lines. *Oncol Rep* 2016; 35: 13–25.
19. Subramaniam D, Ponnurangam S, Ramamoorthy P, et al. Curcumin induces cell death in esophageal cancer cells through modulating notch signaling. *PLoS ONE* 2012; 7: e30590.
20. Liao SK, Xia J, Chen ZW, et al. Inhibitory effect of curcumin on oral carcinoma CAL-27 cells via suppression of Notch-1 and NF-κB signaling pathways. *J Cell Biochem* 2011; 112: 1055–1065.
21. Wang ZW, Zhang YX, Banerjee S, et al. Notch-1 down-regulation by curcumin is associated with the inhibition of cell growth and the induction of apoptosis in pancreatic cancer cells (retracted article, vol. 122, pp. 3247, 2016). *Cancer* 2006; 106: 2503–2513.
22. Zhang H, Xu WL, Li BL, et al. Curcumin promotes cell cycle arrest and inhibits survival of human renal cancer cells by negative modulation of the PI3K/AKT signaling pathway. *Cell Biochem Biophys* 2015; 73: 681–686.
23. Jin H, Qiao F, Wang Y, et al. Curcumin inhibits cell proliferation and induces apoptosis of human non-small cell lung cancer cells through the upregulation of mir-192-5p and suppression of PI3K/Akt signaling pathway. *Oncol Rep* 2015; 34: 2782–2789.
24. Zhao ZM, Li CG, Xi H, et al. Curcumin induces apoptosis in pancreatic cancer cells through the induction of forkhead box O1 and inhibition of the PI3K/Akt pathway. *Mol Med Rep* 2015; 12: 5415–5422.
25. Akkoc Y, Berrak O, Arisan ED, et al. Inhibition of PI3K signaling triggered apoptotic potential of curcumin which is hindered by Bcl-2 through activation of autophagy in MCF-7 cells. *Biomed Pharmacother* 2015; 71: 161–171.
26. Multhaupt HAB, Leitinger B, Gullberg D, et al. Extracellular matrix component signaling in cancer. *Adv Drug Deliv Rev* 2016; 97: 28–40.
27. Berrier AL and Yamada KM. Cell-matrix adhesion. *J Cell Physiol* 2007; 213: 565–573.
28. Hynes RO. The extracellular matrix: not just pretty fibrils. *Science* 2009; 326: 1216–1219.
29. Wozniak MA, Modzelewska K, Kwong L, et al. Focal adhesion regulation of cell behavior. *Biochim Biophys Acta* 2004; 1692: 103–119.
30. Carragher NO and Frame MC. Focal adhesion and actin dynamics: a place where kinases and proteases meet to promote invasion. *Trends Cell Biol* 2004; 14: 241–249.
31. Zhao J and Guan JL. Signal transduction by focal adhesion kinase in cancer. *Cancer Metastasis Rev* 2009; 28: 35–49.
32. Zhao X and Guan JL. Focal adhesion kinase and its signaling pathways in cell migration and angiogenesis. *Adv Drug Deliv Rev* 2011; 63: 610–615.

33. Golubovskaya VM. Survival signaling through focal adhesion kinase in tumors. *Anticancer Agents Med Chem* 2013; 13: 531.
34. Bergamaschi A, Tagliabue E, Sorlie T, et al. Extracellular matrix signature identifies breast cancer subgroups with different clinical outcome. *J Pathol* 2008; 214: 357–367.
35. Liu CC, Lin JH, Hsu TW, et al. Collagen XVII/laminin-5 activates epithelial-to-mesenchymal transition and is associated with poor prognosis in lung cancer. *Oncotarget*. Epub ahead of print 11 August 2016. DOI: 10.18632/oncotarget.11208.
36. Stewart RL, West D, Wang C, et al. Elevated integrin [alpha]6[beta]4 expression is associated with venous invasion and decreased overall survival in non-small cell lung cancer. *Human Pathol* 2016; 54: 174–183.
37. Navab R, Strumpf D, To C, et al. Integrin α11β1 regulates cancer stromal stiffness and promotes tumorigenicity and metastasis in non-small cell lung cancer. *Oncogene* 2016; 35: 1899–1908.
38. Ju LX and Zhou CC. Integrin beta 1 enhances the epithelial-mesenchymal transition in association with gefitinib resistance of non-small cell lung cancer. *Cancer Biomark* 2013; 13: 329–336.
39. Yoon HJ, Cho YR, Joo JH, et al. Knockdown of integrin α3β1 expression induces proliferation and migration of non-small cell lung cancer cells. *Oncol Rep* 2013; 29: 662–668.
40. Ju LX, Zhou CC, Li W, et al. Integrin beta1 over-expression associates with resistance to tyrosine kinase inhibitor gefitinib in non-small cell lung cancer. *J Cell Biochem* 2010; 111: 1565–1574.
41. Elez E, Kocakova I, Hohler T, et al. Abituzumab combined with cetuximab plus irinotecan versus cetuximab plus irinotecan alone for patients with KRAS wild-type metastatic colorectal cancer: the randomised phase I/II POSEIDON trial. *Ann Oncol* 2015; 26: 132–140.
42. Cianfrocca ME, Kimmel KA, Gallo J, et al. Phase 1 trial of the antiangiogenic peptide ATN-161 (Ac-PHSCN-NH_2), a beta integrin antagonist, in patients with solid tumours. *Br J Cancer* 2006; 94: 1621–1626.
43. Besse B, Tsao LC, Chao DT, et al. Phase Ib safety and pharmacokinetic study of volociximab, an anti-α5β1 integrin antibody, in combination with carboplatin and paclitaxel in advanced non-small-cell lung cancer. *Ann Oncol* 2013; 24: 90–96.
44. Kumar A, Dhawan S, Hardegen NJ, et al. Curcumin (Diferuloylmethane) inhibition of tumor necrosis factor (TNF)-mediated adhesion of monocytes to endothelial cells by suppression of cell surface expression of adhesion molecules and of nuclear factor-kappaB activation. *Biochem Pharmacol* 1998; 55: 775–783.