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ORIGINAL ARTICLE

p53-dependent anticancer effects of leptomycin B on lung adenocarcinoma

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Abstract
Purpose Leptomycin B (LMB) and/or its derivatives are considered a novel class of cancer therapeutics through blocking chromosome maintenance region 1, which mediates p53 nuclear export. The objectives of the present study were to first evaluate the cytotoxic effects of LMB on a normal human lung epithelial cell line (BEAS-2B) and three human lung adenocarcinoma cell lines with various p53 status (wild type: A549, mutant: NCI-H522, and null: NCI-H358) and then to identify LMB-induced gene expression alterations in human p53 signaling pathway.
Methods Cells were treated with 0.01–100 nM LMB or 0.1% ethanol (vehicle control) for 4–72 h. Gene expression analyses using gene array for 84 genes involved in p53-mediated signaling pathways were performed in A549 and NCI-H358 after treatment with 20 nM LMB or vehicle control for 24 h.
Results Cytotoxic results from MTS assays revealed a significant dose- and time-dependent effect of LMB on all cell lines. However, this effect was more pronounced in cancer cells than in normal cells, and cancer cells with p53 wild type tended to be less sensitive than those with p53 mutant or null. A total of 23 genes, predominantly involved in apoptosis and cell cycle/proliferation, were significantly altered in A549 after LMB treatment, while no strong modulating effects were observed in NCI-H358. The protein expression of two selected genes, p21 and survivin, was further confirmed by Western blots.
Conclusion Our results suggest that LMB has anti-cancer potential and provides a new regimen of individualized therapy for lung cancer treatment.

Keywords Cytotoxicity · Gene array · Leptomycin B · Lung adenocarcinoma · p53

Introduction

Lung cancer, primarily caused by tobacco exposure, continues to be the leading cause of cancer deaths worldwide and in the United States [1–3]. Furthermore, there are an estimated 116,090 newly diagnosed lung cancer cases (88,900 deaths) in men and 103,350 new cases (70,490 deaths) in women in the United States in 2009 [2]. Non-small-cell lung cancer (NSCLC) remains the predominant form of lung cancer (about 85% of all lung cancers), mainly consisting of the morphological subtypes adenocarcinoma (AC), squamous cell carcinoma (SCC), and large-cell carcinoma (LCC). Lung AC has increased worldwide during the past several decades, becoming the predominant histologic subtype of lung cancer among both men and women [4]. With some improvements in surgical techniques and

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combined therapies over the last several decades, the relative survival rate for lung cancer has slightly increased [5]. However, lung cancer remains extremely lethal with the 5-year survival rate of only about 15% in the United States [1]. Chemotherapy for lung cancer can prolong survival and improve quality of life, but the majority of advanced stage patients die from the disease within 2 years, suggesting that there is still room for improvement [6]. Due to this continual poor prognosis, the development of novel systemic therapy regimens including molecularly targeted therapy has become an important focus of research efforts in lung cancer treatment [7].

The tumor suppressor gene p53 is a key element in apoptosis, cell cycle arrest, and maintaining genomic stability. p53 can activate the transcription of numerous downstream genes (such as p21 and MDM2) by binding to specific sequences, which often mediates their biological functions [8]. Mutations leading to inactivation of p53 (most often in the DNA-binding domain) occur in up to 60% of all types of lung cancer [9, 10] but vary between the different histologic subtypes, with AC generally carrying a lower inactivation mutation rate [11]. It has also been suggested that the p53 protein could be inactivated by degradation or relocalization from the nucleus (cytoplasmic sequestration) in cancer cells with wild-type p53 [12]. One such protein that may relocalize p53 is the nuclear export receptor, chromosome maintenance region 1 (CRM1). CRM1 interacts with leucine/isoleucine-rich nuclear export signals (NESs) to export cancer-associated 'cargo' proteins, including p53 [13, 14]. In addition, alterations or defects in nuclear-cytoplasmic transport have been observed in different cancer cells involved in various pathways including p53-mediated signal transduction pathways [15]. For instance, recent studies have showed that CRM1 has significantly higher expression in cancers of the cervix [16], brain [17], and pancreas [18] when compared to normal tissues. Finally, inhibition of CRM1 in cancer cells significantly decreases cell proliferation, partially related to apoptosis by increasing p53 expression [16]. Combined, these previous studies have suggested targeting of the nuclear export receptor CRM1 as a new lung cancer treatment regimen.

Leptomycin B (LMB) is a metabolite from *Streptomyces* sp. strain ATS1287, which historically serves as an antifungal antibiotic. LMB, a highly specific and potent inhibitor of CRM1, can induce the eukaryotic cell elongation and cell cycle arrest at the G1 and G2 phases [19, 20]. It has antitumor activity by irreversibly reacting with a cysteine residue near or within the cargo-binding domain of CRM1, which inhibits the NES-dependent nuclear export function [21–23]. LMB treatment has been shown to induce cell death and p53 nuclear accumulation in esophageal cancer cells [24], to induce the cell death in cervical carcinoma cell lines by nuclear sequestration of p53 and reactivation of p53-dependent transcription [25], and to increase p53 protein levels and transcriptional activity in prostate cancer cells [26]. It has also been shown that LMB can augment the therapeutic effects of p53 gene therapy [24].

The potential of LMB as an anti-lung cancer agent and the possible mechanism and function of LMB inhibiting CRM1 with p53 involvement in lung cancer have not been fully investigated. The objectives of the present study are to evaluate cytotoxicities of LMB on a normal lung epithelial cell line and three lung cancer cell lines with different p53 status (wild type, mutant and null), and to probe possible mechanisms by identifying alterations of gene expression related to p53 signaling pathways.

Materials and methods

Cell culture

To favor adhesion of the human bronchial epithelial cell line BEAS-2B, the flasks/plates/dishes (Corning Incorporated Life Sciences, Lowell, MA) were coated with a mixture of 0.01 mg/mL fibronectin (Sigma, St. Louis, MO), 0.03 mg/mL bovine collagen type I (Inamed biomaterials, Freemont, CA), 0.01 mg/mL bovine serum albumin (Sigma), and bronchial epithelial cell basal medium (BEBM, Lonza, Walkersville, MD) overnight at 37°C. Bronchial epithelial cell growth medium (BEGM, Lonza) was supplemented with 2 mL bovine pituitary extract, 0.5 mL insulin, 0.5 mL hydrocortisone, 0.5 mL retinoic acid, 0.5 mL transferrin, 0.5 mL triiodothyronine, 0.5 mL epinephrine, and 0.5 mL human epidermal growth factor. BEAS-2B cells were maintained in BEGM supplied with 50 U/mL penicillin and 50 µg/mL streptomycin (Invitrogen Corporation**,** Carlsbad, CA). The AC cell lines A549 (p53 wild type), NCI-H522 (p53 mutant, exon 6 codon 191 CCT → CT), and NCI-H358 (p53 null) were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 µg/mL streptomycin. All cells were cultured at 37°C in a humidified incubator with 95% of air and 5% of CO_2 by volume. The p53 mutations of these cell lines have been confirmed by polymerase chain reaction-single strand conformation polymorphism (data not shown).

Cell viability assay

Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, Promega, Madison, WI) assay as previously described [27]. Briefly, cells were plated at a density of 1×10^4 cells for BEAS-2B and 5×10^3 cells for all the lung cancer cell lines per well in 96-well plates. Vehicle control (0.1% ethanol) or LMB (LC Labs, Woburn,

MA) was added at the concentrations of 0.01–100 nM for 4, 12, 24, 48, and 72 h. Three hours before the end of each time point, 20 μL of a mixture containing 333 μg/mL MTS and 25 μM phenazine methosulfate (PMS, Sigma) was added to each well and incubated at 37°C. At the various time points, the cell viability was determined by measuring the absorbance at 490 nm using a SpectraMax Plus Spectrophotometer (Molecular Devices, Sunnyvale, CA). Six replicates at each concentration and time point were analyzed. The experiments were performed independently in duplicate. Vehicle-treated controls and blanks were incubated in the same plate under the same conditions.

Isolation of total RNA

Total RNA was isolated from cells with/without LMB treatment using an RNeasy® plus mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Briefly, 3×10^6 A549 or NCI-H358 cells were treated with 20 nM LMB or vehicle control for 24 h and lysed. The lysate was homogenized, genomic DNA removed, and purified. The RNA was finally eluted by 30 μL RNase-free water and stored at −80°C until use. The yield of extracted total RNA was determined by measuring OD at 260 nm using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA). The quality and purity of total RNA were evaluated by agarose gel electrophoresis using Horizon 11.14 (Life Technologies, Gaithersburg, MD). All total RNA samples used for RT–PCR experiments had good integrity and had OD A260/A280 ratios between 1.9–2.1 and concentration ≥4 μg/mL. The total RNA samples were further treated by DNA-free™ DNase (Applied Biosystems, Austin, TX) to remove possible DNA contamination.

Reverse transcription

cDNA was prepared using an RT^2 PCR array first strand kit (SABiosciences Corporation, Frederick, MD) according to manufacturer's instructions. Briefly, 1 μg of total RNA was mixed with 2 μL of 5× *g*DNA elimination buffer to form a total volume of 10 μL genomic DNA elimination mixture with RNase-free H_2O, incubated at 42°C for 5 min, and chilled on ice immediately. The mixture was then incubated with a 10 μL RT Cocktail at 42°C for 15 min, and the reaction stopped by heating at 95°C for 5 min to inactivate the reverse transcriptase. The 20 μL cDNA synthesis reaction mixture was diluted to 111 μL by adding 91 μL RNase-free H_2O and kept on ice for further use.

Array-based SYBR® green RT–PCR

Constitutive gene expression profiling was performed using the RT^2 Profiler™ PCR array (SABiosciences) related to p53-mediated signal transduction based on manufacturer's instructions. The gene array profiled the expression of 84 genes involved in apoptosis, cell cycle, cell growth, proliferation and differentiation, and DNA repair. In addition, the array included the controls for human genomic DNA contamination, reverse transcription, positive PCR control, and 5 housekeeping genes [beta-actin (ACTB), beta-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1), and ribosomal protein L13a (RPL13A)] (Supplementary Tables 1 and 2). Briefly, an aliquot of 102 μL diluted cDNA synthesis reaction was mixed with an experimental cocktail containing 1,275 μL 2 × RT^2 SYBR® green/fluorescein qPCR master mix (SABiosciences) and 1,173 μL Mili-Q water (18.3 M, pH 6.8) to form the PCR master mixture. An aliquot of 25 μL of the mixture (a total of 9.0 ng cDNA) was added to each well of the 96-well PCR array. Real-time PCR were performed using a two-step cycling program on an ABI PRISM 7000 System (Applied Biosystems) under the following conditions: 10 min at 95°C (cycle 1) followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. SYBR® green fluorescence was detected and recorded from each well during the annealing step of each cycle. The threshold cycle (C_T) above the background for each reaction was calculated. Experiment was performed in duplicate for both treated and control groups.

Western blot

A549 and NCI-H358 cells were treated with 20 nM LMB or vehicle control for 24 h and lysed in a Radio Immuno-Preciptation Assay (RIPA) lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) on ice for 30 min. Protein concentrations were measured using the Bio-Rad Bradford protein assay (Bio-Rad, Hercules, CA). After boiling for 5 min, lysates containing 30 μg of protein per well were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes, followed by incubating overnight at 4°C in blocking buffer containing 3% milk in 1 × phosphate-buffered saline (PBS) and 0.1% Tween 20 (1 × PBST). Thereafter, the membranes were incubated for 1 h with primary antibodies (anti-p21 or anti-survivin, Santa Cruz Biotechnology) at a 1:1,000 dilution and for 1 h with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibodies (GE Healthcare, Piscataway, NJ) at a dilution of 1:2,000 at room temperature. After briefly incubating with enhanced chemiluminescence reagents (ECL, GE Healthcare), the signals on membranes were exposed to X-ray films (Fujifilm Corporation, Tokyo). Densitometric digital analysis of protein bands was performed to quantify each protein band using Quantity

One 1-D Analysis Software version 4.1.0 (Bio-Rad). The experiments were performed independently in triplicate. Each protein was normalized by the intensity of the housekeeping gene α-tubulin from each sample.

Statistical analyses

The percentage of viable cells in each well relative to control group (100% viable) was calculated and plotted against the concentration of LMB by SigmaPlot 10.0 (Systat Software Inc., San Jose, CA). A non-linear regression curve fit was applied, and the 50% inhibitory concentrations (IC50s) were calculated using Graphpad Prism 5.0 software (GraphPad Software, La Jolla, CA). The IC50s were compared between normal and cancer cell lines and among lung cancer cell lines with different p53 status by Wilcoxon rank sum test. Data from RT–PCR were quantified by the ABI sequence detection software and normalized by 5 housekeeping genes (endogenous control). ΔC_T was defined as the value of subtracting the C_T value of endogenous control from the C_T value of the target messenger RNA (mRNA). The fold change for treated sample relative to the control sample could be obtained by $2^{-(\Delta CT\ treat)}/2^{-(\Delta CT\ control)}$ and compared. *P* value less than 0.05 based on *t*-test and a mean difference equal to or greater than twofold were used as the criteria to determine the differentially expressed genes. Protein expression levels of p21 and survivin between LMB-treated group and vehicle-treated control group were compared by one-way ANOVA. The fold changes for treated sample relative to the control sample were also compared. The comparisons for data on IC50s and protein expression were performed using the SPSS 13.0 software. Differences with $P < 0.05$ were considered significant.

Results

Cytotoxicities of LMB on lung cell lines

The cytotoxicities of LMB were evaluated on the normal bronchial epithelial cell line BEAS-2B and the three lung cancer cell lines, including A549, NCI-H522, and NCI-H358, by treatment with vehicle control (0.1% ethanol) or 0.01–100 nM LMB for 4, 12, 24, 48, and 72 h. Inhibitory effects of LMB, as measured by MTS cell proliferation assay, were dose- and time-dependent for all four cell lines ($P < 0.001$) (Figs. 1, 2). The IC50s could not be extrapolated for all four cell lines at 4, 12, and 24 h after LMB treatment (Figs. 1, 2). The IC50s for BEAS-2B were 18.0 nM at 48 h and 4.5 nM at 72 h after LMB treatment (Table 1; Fig. 1). The IC50s for BEAS-2B were significantly higher than the median IC50s for the three lung cancer cell lines, which were 6.4 (range: 0.5–13.1) and 2.0 (range: 0.4–3.4) nM at 48 and 72 h, respectively ($P < 0.005$) (Table 1; Figs. 1, 2). In addition, lung cancer cells with p53 wild type tended to be less sensitive than those with p53 mutant or null ($P < 0.001$) (Table 1; Fig. 2). The IC50s were 13.1 nM and 3.4 nM at 48 h and 72 h for cells with wild-type p53 (A549). For NCI-H522 cells (p53 mutant), the IC50s of LMB were 5.7 nM at 48 h and 2.2 nM at 72 h. For NCI-H358 cells (p53 null), the IC50s were 0.5 nM at 48 h and 0.4 nM at 72 h. Cell viability in the presence of the medium alone or the 0.1% ethanol vehicle was equivalent.

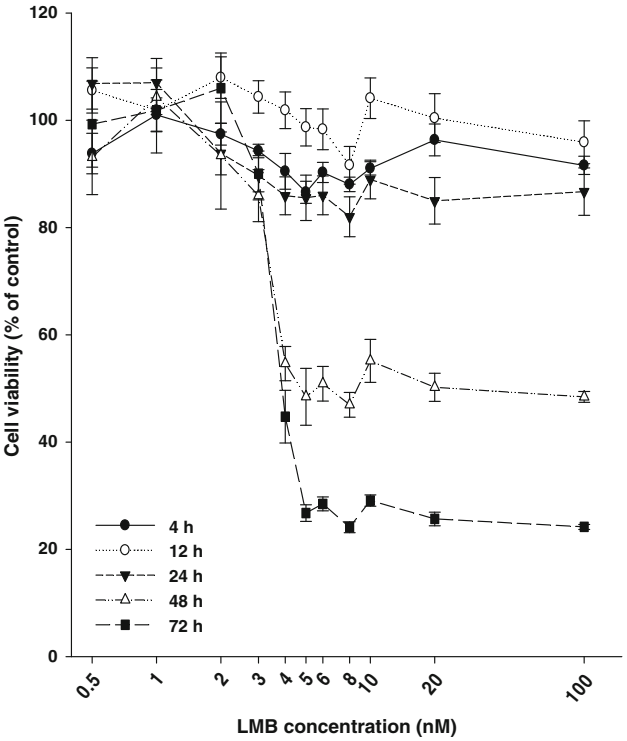

Fig. 1 The effects of LMB on BEAS-2B at 4, 12, 24, 48, and 72 h as determined by the MTS assay. Cells were treated with 0.01–100 nM LMB for 4–72 h. Data were expressed as a percentage of vehicle control (0.1% ethanol). LMB concentration was shown as horizontal axis with a log10 scale. Values were represented as means ± SD, $n = 6$

Gene expression alterations after LMB treatment

Based on p53 status and LMB sensitivity, we selected the cell lines A549 (p53 wild type, most resistant) and NCI-H358 (p53 null, most sensitive) to investigate the effect of 20 nM LMB treatment for 24 h on genes related to p53 signaling pathway. The expression patterns of 84 genes involved in the p53 pathway were screened (Supplementary Tables 1 and 2). The duplicate samples from each group produced reproducible results. By comparing to the reaction of negative control, 12 genes were assigned as undetectable in A549 and 15 genes were assigned as

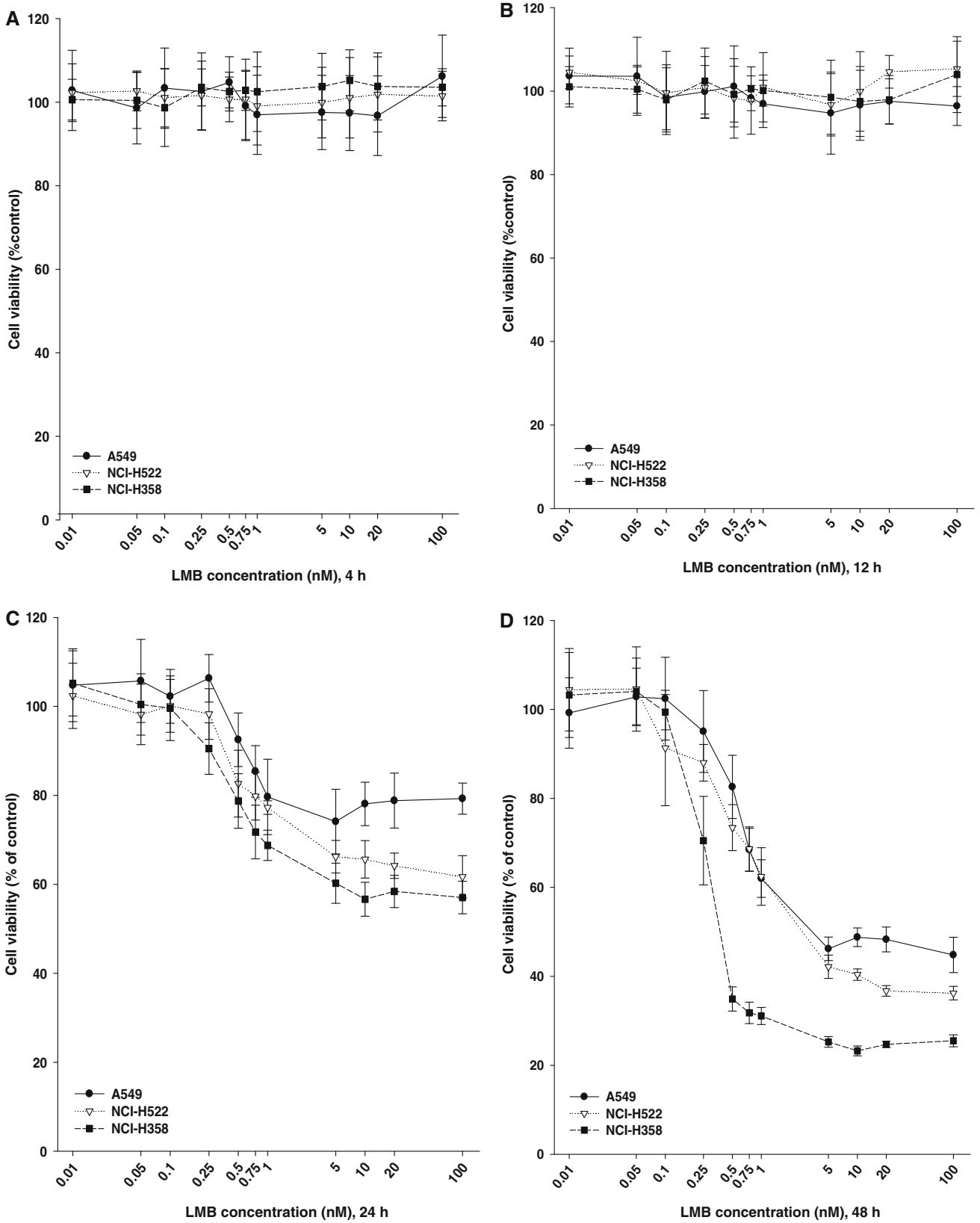

Fig. 2 The effects of LMB on three lung cancer cell lines at 4 (**a**), 12 (**b**), 24 (**c**), 48 (**d**), and 72 h (**e**) as determined by the MTS assay. Cells were treated with 0.01–100 nM LMB for 4–72 h. Data were expressed as a percentage of vehicle control (0.1% ethanol). LMB concentration was shown as horizontal axis with a log10 scale. Values were represented as means ± SD, $n = 6$

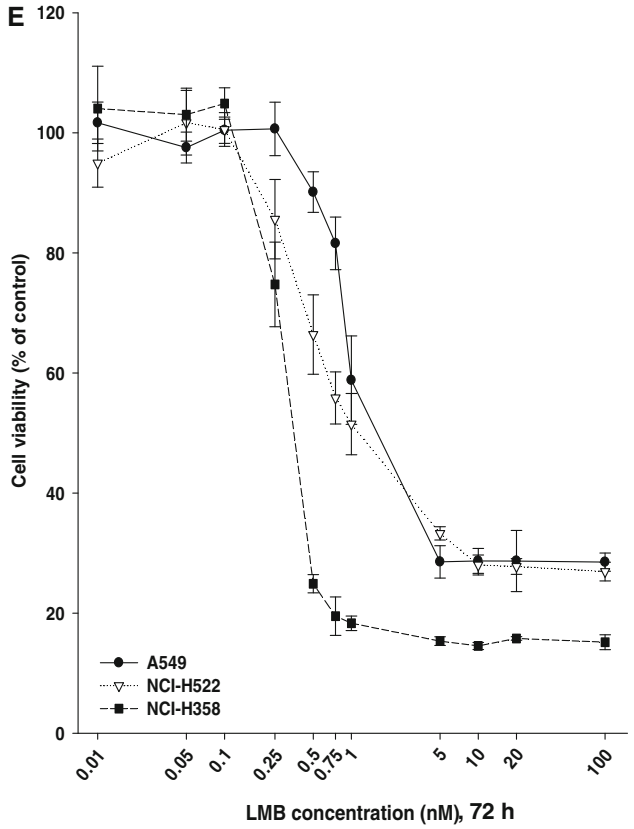

Fig. 2 continued

Table 1 Cytotoxicity of LMB on BEAS-2B and three lung cancer cell lines

Time point	Cell lines	p53	IC50s
48 h	BEAS-2B	Wild type	18.0
	A549	Wild type	13.1
	NCI-H522	Mutant	5.7
	NCI-H358	Null	0.5
72 h	BEAS-2B	Wild type	4.5
	A549	Wild type	3.4
	NCI-H522	Mutant	2.2
	NCI-H358	Null	0.4

undetectable in NCI-H358. Among 72 determined genes in A549 cells, the coefficient of variation (CV) was 1.0% for the control sample and 0.6% for the LMB-treated sample. Among 69 determined genes in NCI-H358 cells, the CV was 1.3% for the control sample and 0.7% for the LMB-treated sample (Supplementary Tables 1 and 2).

This analysis showed the expression of 13 genes decreased more than twofold in A549 cells treated with LMB when compared to vehicle controls, including apoptotic genes: baculoviral inhibitor of apoptosis (IAP) repeat-containing 5 (BIRC5, survivin) and tumor protein p63 (TP63); cell cycle-related genes: cyclin B2 (CCNB2), cyclin E2 (CCNE2), cell division cycle 2 (CDC2), cell division cycle 25 homolog A (CDC25A), CHK1 checkpoint homolog (CHEK1), E2F transcription factor 1 (E2F1), and protein regulator of cytokinesis 1 (PRC1); cell proliferation-related genes: cell division cycle 25 homolog C (CDC25C) and pituitary tumor-transforming 1 (PTTG1); and DNA repair genes: breast cancer 1 (BRCA1) and DNA (cytosine-5-)-methyltransferase 1 (DNMT1) (Fig. 3a). In contrast, 10 genes increased more than twofold in LMB-treated A549 cells when compared to controls, including apoptotic genes: etoposide-induced 2.4-kb transcript (EI24), leucine-rich repeats and death domain containing (LRDD), tumor necrosis factor receptor superfamily member 10b (TNFRSF10B), and TNFRSF10D; cell cycle-related genes: cyclin-dependent kinase inhibitor 1A (CDKN1A, p21), E2F transcription factor 3 (E2F3), growth arrest and DNA damage-inducible alpha (GADD45A), and sestrin 1 (SESN1); and cell proliferation-related genes: B-cell translocation gene family member 2 (BTG2) and Mdm2 p53-binding protein homolog (mouse) (MDM2) (Fig. 3a; Supplementary Table 1).

For NCI-H358 cells, the 7 down-regulated genes with LMB treatment when compared to controls were apoptotic gene: TP63; cell cycle-related genes: CHEK2 and hexokinase 2 (HK2); cell proliferation-related genes: MDM4 and protein kinase C alpha (PRKCA); and DNA repair genes: BRCA1 and DNMT1. The 5 up-regulated genes with LMB treatment when compared to controls were apoptotic genes: apoptotic peptidase activating factor 1 (APAF1), nuclear factor (NF) κB1, survivin, and TNFRSF10D; and cell proliferation-related gene: interleukin 6 (IL6) (Fig. 3b; Supplementary Table 2).

The differences in gene expression between A549 and NCI-H358 cells

Among the 84 genes analyzed, before LMB treatment, expressions of 6 genes were only detected in A549 but not in NCI-H358, including brain-specific angiogenesis inhibitor 1 (BAI1), B-cell CLL/lymphoma 2 (BCL2), BTG family member 2 (BTG2), early growth response 1 (EGR1), tumor protein p53 (TP53), and Wilms tumor 1 (WT1). On the other hand, expressions of 3 genes were only detected in NCI-H358 but not in A549, including cyclin-dependent kinase inhibitor 2A (CDKN2A), IL6, and reprimo TP53-dependent G2 arrest mediator candidate (RPRM).

Among 66 genes detectable in both A549 and NCI-H358, 11 genes showed more than twofold higher expression in A549 vs. NCI-H358 cells before LMB treatment, including BCL2-associated X protein (BAX), caspase 9 apoptosis-related cysteine peptidase (CASP9), GADD45A, insulin-like growth factor 1 receptor (IGF1R), lysine

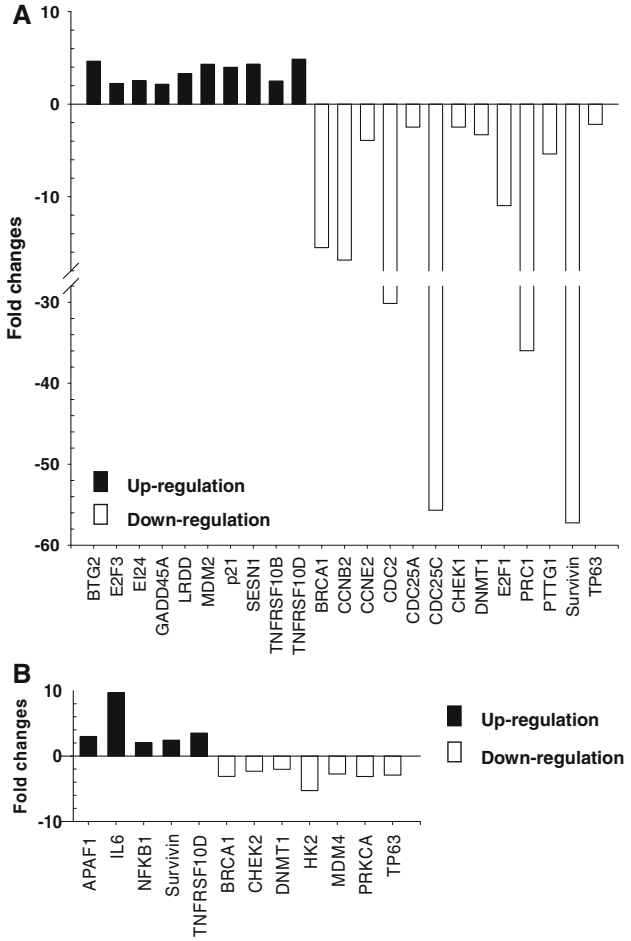

Fig. 3 The alterations of gene expression by RT–PCR array in A549 (**a**) and NCI-H358 (**b**) after LMB treatment. In comparison with untreated cells, the altered genes in LMB-treated A549 and NCI-H358 cells were profiled, and the *black bars* showed genes up-regulated by LMB, while the *white bars* represented down-regulation

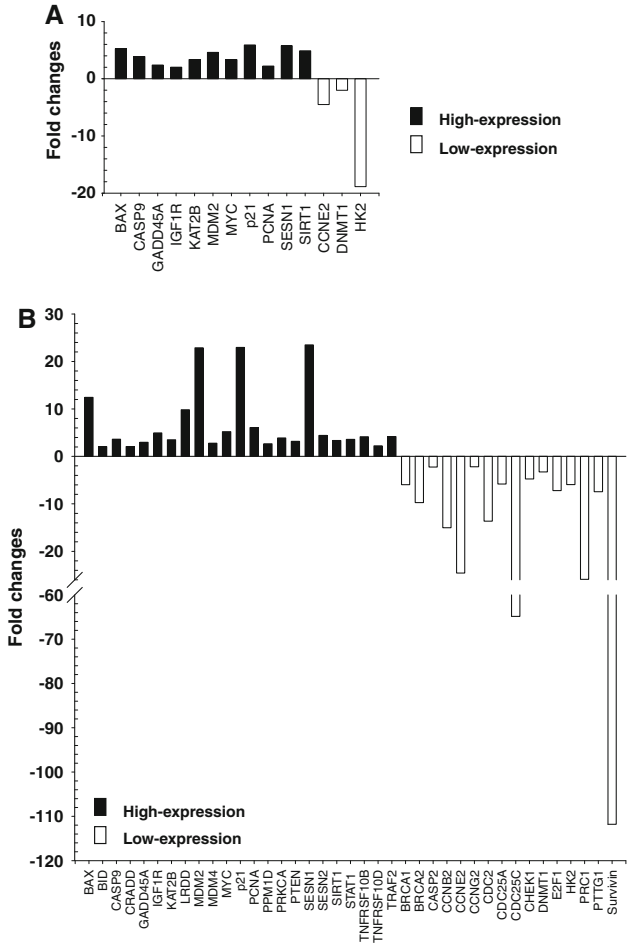

Fig. 4 Gene expression with twofold differences in A549 cells before LMB treatment (**a**) and after LMB treatment (**b**) in comparison with NCI-H358 cells. Genes with higher expression in A549 cells were shown with *black bars*, and genes with lower expression in A549 cells were presented with *white bars*

acetyltransferase 2B (KAT2B), MDM2, V-myc myelocytomatosis viral oncogene homolog (avian) (MYC), p21, proliferating cell nuclear antigen (PCNA), SESN1 and sirtuin 1 (SIRT1) (Fig. 4a). On the other hand, 3 genes showed significantly higher in NCI-H358 vs. A549, including CCNE2, DNMT1, and HK2 (Fig. 4a).

After LMB treatment, 23 genes showed more than twofold higher expression in A549 vs. NCI-H358 cells, including BAX, BH3-interacting domain death agonist (BID), CASP9, CASP2 and RIPK1 domain containing adaptor with death domain (CRADD), GADD45A, IGF1R, KAT2B, LRDD, MDM2, MDM4, MYC, p21, PCNA, protein phosphatase 1D magnesium-dependent (delta isoform) (PPM1D), PRKCA, phosphatase and tensin homolog (PTEN), SESN1, SESN2, SIRT1, signal transducer and activator of transcription 1 (91 kDa) (STAT1), TNFRSF10B, TNFRSF10D, and TNF receptor-associated factor 2 (TRAF2) (Fig. 4b). On the other hand, 16 genes showed higher expression in NCI-H358 vs. A549, including BRCA1, BRCA2, CASP2, CCNB2, CCNE2, CCNG2, CDC2, CDC25A, CDC25C, CHEK1, DNMT1, E2F1, HK2, PRC1, PTTG1, and survivin (Fig. 4b).

p21 and survivin expression

p21 and survivin were selected for protein analyses based on their well-known status as p53 downstream targets as well as different modulating effects between A549 and NCI-H358 after LMB treatment. The relative protein expression levels of p21 were 0.22 ± 0.02 and 0.44 ± 0.02 in cells treated with vehicle control and 20 nM LMB (LMB vs. control: $P < 0.001$, Fig. 5). This data indicated that the protein expression of p21 was increased twofolds in A549 cells treated with 20 nM LMB for 24 h in comparison with control cells. In addition, survivin was dramatically down-regulated in A549 cells treated with 20 nM LMB for 24 h,

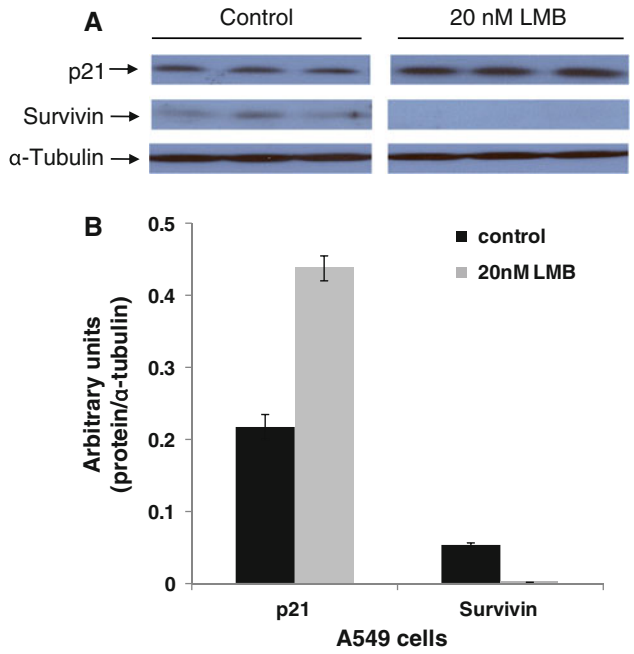

Fig. 5 Effects of LMB on the protein expression of p21 and survivin in A549. **a** A549 cells were cultured in the presence of the vehicle control (0.1% ethanol) or 20 nM LMB for 24 h. Western blot was then performed to determine the protein expression levels of p21 and survivin. **b** The relative protein intensity of p21 and survivin in A549 after 24 h of LMB treatment when compared to the intensity of α-tubulin. Experiment was done in triplicate. The intensity of each band was quantified using Quantity One software. Data represents the means ± SD

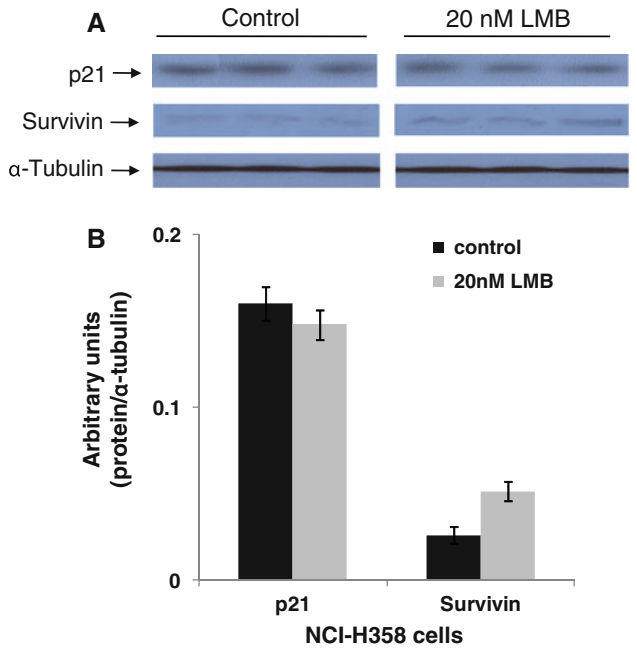

Fig. 6 Effects of LMB on the protein expression of p21 and survivin in NCI-H358. **a** NCI-H358 cells were cultured in the presence of vehicle control (0.1% ethanol) or 20 nM LMB for 24 h. Western blot was then performed to determine the protein expression levels of p21 and survivin. **b** The relative protein intensity of p21 and survivin in NCI-H358 after 24 h of LMB treatment when compared to the intensity of α-tubulin. Experiment was done in triplicate. The intensity of each band was quantified using Quantity One software. Data represents the means ± SD

which was only detected in control A549 cells, but not in the LMB-treated group. The relative protein expression levels of survivin were 0.05 ± 0.00 and 0.00 ± 0.00 in cells treated with vehicle control and 20 nM LMB (LMB vs. control: $P < 0.001$, Fig. 5).

On the other hand, for NCI-H358 cells, the relative protein expression levels of p21 were 0.16 ± 0.01 in vehicle-treated control cells and 0.15 ± 0.01 in 20 nM LMB-treated cells (LMB vs. control: $P = 0.178$) (Fig. 6). In addition, the relative protein expression levels of survivin were 0.03 ± 0.01 and 0.05 ± 0.01 in cells treated with vehicle control and 20 nM LMB (LMB vs. control: $P = 0.004$) (Fig. 6). Thus, the protein expression of survivin was up-regulated around twofolds, while no significant protein expression alteration of p21 was observed in NCI-H358 cells after LMB treatment in comparison with those of controls (Fig. 6).

Discussion

We hypothesized in the present study that LMB would have the anti-tumor activity on lung cancer, and the targeted therapy effects of LMB on lung cancer are p53-dependent. Our results showed that the cell growth inhibitory effects of LMB were dose- and time-dependent for all four human lung cell lines. We also found that lung cancer cells exhibited more inhibitory effects when compared to normal bronchial epithelial cells BEAS-2B. In addition, this study showed that lung cancer cells with the p53 wild type were less sensitive than those with the p53 mutant or null. Finally, LMB modulated p53-responsive genes with significantly different profiles when comparing p53 wild type to p53 null cell lines.

We found in this study that more pronounced inhibitory effects of LMB occurred in lung cancer cells when compared to normal bronchial epithelial cells BEAS-2B, which suggested the anti-tumor activity of LMB on lung cancer cells. These findings are in line with previous results which showed that LMB had a relatively mild and reversible effect by inducing growth arrest at mM concentrations in human normal fibroblasts, while the cytotoxic effects of LMB on human neuroblastoma cell lines were stronger, even at nM concentrations [28]. Among the three NSCLC cell lines tested, the cytotoxic effects of LMB were significantly dose- and time-dependent with median IC50s of 6.4 and 2.0 nM at 48 and 72 h, respectively. This is in agreement with previous studies which found that LMB was very toxic to a wide variety of cancer cell lines with IC50s of

0.1–10.0 nM, and LMB induced cell death in a dose-dependent manner [14, 24].

In the present study, lung cancer cells with p53 wild type were less sensitive than those with p53 mutant or null. For instance, the IC50s for A549 (p53 wild type) were 13.1 and 3.4 at 48 and 72 h, while they were 0.5 and 0.4 at 48 and 72 h for NCI-H358 cells (p53 null). The cytotoxic data of LMB on three additional lung cancer cell lines, including a squamous cell carcinoma cell line NCI-H226 (p53 mutant), a large-cell carcinoma cell line NCI-H460 (p53 wild type), and an adenosqumous carcinoma cell line NCI-H596 (p53 mutant), have confirmed that LMB is less cytotoxic on cell lines with p53 wild type than those with p53 mutants (data not shown). Activated p53 has been shown to be associated with either increased sensitivity or resistance to the cytotoxicity of cancer therapeutic agents [29, 30]. For instance, the cytotoxic activity of LMB was more pronounced in esophageal cancer cell lines with p53 wild type (TE2) than cells with p53 mutant (T.Tn, codon 213 CAT → CGT) or p53 null (TE3) [24]. It has been suggested that p53 potentiates apoptosis in response to severe DNA damage, which could override its response on G1 checkpoint [31–33], and the restoration of wild-type p53 enhances the sensitivity of cells to DNA damage-induced agents [31, 34]. On the contrary, wild-type p53 activity of cell growth arrest and facilitating DNA repair could increase the resistance to radio- or chemotherapeutic agents inducing DNA damage [35, 36]. For instance, loss of normal p53 function increased sensitization of normal human fibroblasts to the anticancer agent, paclitaxel, which may result from an increase of G2/M phase arrest, micronucleation, and p53-independent apoptosis [37]. We found a more pronounced inhibitory effect of LMB on lung cancer cells than normal bronchial epithelial cells BEAS-2B (p53 wild type) and also suggested the possible resistance of wild-type p53 cells in response to LMB treatment. Finally, p53-independent cytotoxicities and apoptosis were observed with the treatment of radiation or chemical agents, and resistant effects may be due to factors or pathways other than p53 [33, 38, 39]. For instance, a recent gene expression profiling study showed that arsenic induced cell death through a p53-independent pathway in p53-deficient cells [39]. This could also explain the cytotoxic effects we observed in NCI-H358 in the present study. Collectively, although a clear mechanism between p53 status and cell susceptibility to anticancer therapeutic agents is difficult to establish, the different inhibitory effects of LMB on lung cancer cell lines with different p53 status found in the present study suggest the importance of targeted therapy in response to LMB.

Loss of control of genomic stability is central in the development of cancer, and p53 is a key element in maintaining genomic stability [40]. The RT^2 Profiler PCR Array allows profiling multi-genes simultaneously by combining RT–PCR and microarrays [41], and this approach has become a convenient and reliable technology to perform high-throughput screening of alterations in gene expression [42]. We have applied this method to profile expression of 84 genes related to p53-mediated signal transduction in A549 or NCI-H358 cells with/without LMB. Among 13 down-regulated genes in A549 cells after LMB treatment, CDC2, CDC25C, PRC1, and survivin decreased more than tenfold in comparison with those in control cells. CDC2, CDC25C and PRC1 play an essential role in regulating cell division, overexpression in cancers, and are negatively regulated by p53 [43, 44]. CDC2 and CDC25C were reported to be down-regulated in cells with p53 wild type upon doxorubicin-mediated DNA damage [44]. Survivin can inhibit apoptosis, enhance proliferation and promote angiogenesis and was reported to be negative regulated by wild-type p53 and involved in p53-dependent apoptotic pathway [45]. We found that the expression of survivin showed remarkable consistent down-regulation at both transcription and protein levels in A549 cells (p53 wild type) but slight up-regulation in NCI-H358 cells (p53 null) after LMB treatment. Down-regulation of survivin or inactivation of its function has been shown to suppress tumor growth in several animal studies [46]. Moreover, nuclear export of survivin is CRM1-mediated and essential for its tumor-promoting biological activity [47]. Therefore, LMB may directly or indirectly modulate the expression of survivin to deactivate its function. Altogether, the down-regulation of gene expression in a group of genes involved in apoptosis and cell growth suppression observed after LMB treatment in A549 could reflect the cytotoxic effect of LMB.

In addition, ten up-regulated genes in LMB-treated A549 cells were closely associated with p53 signal pathways, such as BTG2, MDM2, p21, SESN1, and TNFRSF10D. Under physiological conditions, p53 and MDM2 forms a critical auto-regulatory feedback loop to keep p53 at low levels [48]. Altered p53 modification state could interfere with its interaction with MDM2 and facilitate p53 stabilization, which in turn increase the expression of p53 downstream genes [48, 49]. The up-regulation of MDM2 in A549 cells after LMB treatment found in the present study might be due to the feedback of increased p53 level/activity. p21 plays a primary role in cell cycle arrest and is involved in apoptosis [8, 50]. p53 triggers cell cycle arrest based on its downstream genes, such as p21 through the modulation of cyclin-dependent kinases (CDK) [51]. Our study presented that the expression of p21 was increased consistently at both transcription and protein levels in A549 cells but not NCI-H358 cells after LMB treatment. These are in line with results from several studies which showed that p21 mRNA was increased by LMB treatment in human normal primary fibroblasts [52], and the increased p21 protein expression was observed in

esophageal cancer cells with p53 wild type, but not in esophageal cancer cells with p53 null [24]. Several studies have shown DNMT1, an important component of oncogenic pathway, could repress $p21^{WAF1}$ transcription with an inverse relationship of expression between DNMT1 and $p21^{WAF1}$ [53, 54]. In line with this, the expression of DNMT1 in A549 cells was decreased. Finally, BTG2, SESN1, and TNFRSF10D are negative regulators of cell proliferation and considered as promising anticancer targets [55–57]. Taken together, the gene expression data from A549 suggest p53-mediated genes play important roles in cellular chemosensitivity to LMB. This is also supported by other studies that suggest the inhibitory effects of LMB on tumor cells through various mechanisms, including cell cycle arrest, apoptosis, and cytotoxicity [20, 58, 59].

For NCI-H358 cells, only 2 genes, HK2 and IL6, altered dramatically after LMB treatment, suggesting that LMB treatment did not show strong modulating effects on p53-mediated genes in NCI-H358 (p53 null). HK2 is a predominant isoform of hexokinase that is overexpressed in malignant tumors [60]. The function of HK2 is based on its subcellular localization; it is a key enzyme in glycolysis in cytoplasm, but is also a factor related to glucose repression signaling of several Mig1-regulated genes in nucleus [61, 62]. In a highly glycolytic tumor, a majority of HK2 is localized in the nucleus, and the interaction of HK with mitochondria plays an important role in not only maintenance of a high glycolytic rate in malignances but also regulation of the cell apoptotic signaling cascades, which is crucial for tumor survival [61]. The nuclear export of HK2 is CRM1-dependent [62], and inhibitor of HK2 had an antitumor effect through triggering apoptosis [63]. IL6, a pro-inflammatory cytokine involved in the regulation of immune and inflammatory responses, has been identified as an important growth modulator of a subset of human lung cancer cells by increasing cell proliferation and inhibiting apoptosis [64]. The involvement of IL6 in cytotoxicity of LMB on NCI-H358 is unclear. However, IL6 is recently reported to induce apoptosis in STAT3-depleted cells [65] and in normal fibroblasts [66]. The data from NCI-H358 suggest LMB treatment did not show strong modulating effects on p53-mediated genes in this cell, which is as expected since NCI-H358 is a cell with p53 null. Nevertheless, some of the gene expression data are speculative, and further experiments are needed to substantiate the functional involvement of the particular genes after LMB treatment in both A549 and NCI-H358.

A549 cells were less sensitive to LMB treatment than NCI-H358. These phenomena may be partially due to the fact that A549 and NCI-H358 have different molecular signatures. For instance, among the 84 genes we screened, some genes are uniquely expressed in A549, such as p53, or NCI-H358, such as IL6, and some genes had significantly different expression between A549 and NCI-H358 cells, such as BAX, CCNE2, HK2, MDM2, p21, and SESN1. In addition, the different response profiles after LMB treatment between these two cells may contribute to the distinct responses of cells to LMB. Our data suggested that the cell growth inhibition effects of LMB on A549 could be closely related to p53-induced cell cycle arrest and/or apoptosis. On the other hand, unlike A549, LMB treatment did not show strong modulating effects on p53-mediated genes in NCI-H358. The more pronounced inhibition of cell growth of LMB on NCI-H358 when compared to A549 could result from signal transduction pathways other than p53. Altogether, these findings suggest the involvement of p53 in cellular response of A549 to LMB but not NCI-H358, and LMB could eliminate cancer cells probably in distinct mechanisms for cancer cells with different p53 status. Our findings in lung cancer cell lines disagreed with the finding from the cytotoxic data in esophageal cancer cell lines, [24]. In addition, a recent study examined the potential of LMB derivatives serving as the paradigm for a novel class of cancer therapeutics, suggesting activated p53 are the key element for responses [14]. These discrepancies have to be further investigated.

In summary, our results showed that LMB inhibits cell proliferation in a dose- and time-dependent manner. The abilities of LMB leading to remarkable cell growth inhibition and/or apoptosis in lung cancer cells but not normal cells make LMB and/or its derivatives a promising drug in lung cancer treatment. The different cellular responses of cells with distinct p53 status suggest the importance of individualized therapy. Future studies involved in more cell lines, animal models, and clinical samples are needed to confirm these findings. Further investigations into possible molecular mechanisms of nuclear export inhibitors, such as LMB and/or its derivatives, as anti-cancer reagents are warranted.

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