RESEARCH ARTICLE

Curcumin reduces pulmonary tumorigenesis in vascular endothelial growth factor (VEGF)-overexpressing transgenic mice

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Scope: We investigated the inhibition of pulmonary tumor formation through treatment with curcumin in transgenic mice.

Methods and results: In this study, a strain of transgenic mice carrying human vascular endothelial growth factor A_{165} (*hVEGF-*A_{165}) gene to induce pulmonary tumor was used as an in vivo cancer therapy model. We found that curcumin significantly reduced hVEGF-A_{165} overexpression to normal, specifically in Clara cells of the lungs of transgenic mice, and suppressed the formation of tumors. In addition, we demonstrated a relationship between curcumin treatment and the expression of VEGF, EGFR, ERK2, and Cyclin A at the transcriptional and translational levels. We also noticed a reduction of Cyclin A and Cyclin B after curcumin treatment that had an effect on the cell cycle. Curcumin-induced inhibition of Cyclin A and Cyclin B likely results in decreased progression through S and G2/M phases. These results demonstrated that the expression of proteins involved in the S to M phase transition in transgenic mice is suppressed by curcumin.

Conclusion: A Data suggest that a blockade of the cell cycle may be a critical mechanism for the observed effects on vasculogenesis and angiogenesis following treatment with curcumin.

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Curcumin / Pulmonary cancer / Transgenic mice / Turmeric / VEGF

1 Introduction

According to statistics supplied by the Taiwanese Department of Health, cancers have ranked as leading causes of death in Taiwan since 1982. There are more than 30 000 people diagnosed with cancer every year, and thus, cancer has become the largest health threat. Of the top ten lethal cancers, pulmonary cancer ranked first in the past 10 years in Taiwan. Although the treatment of pulmonary cancer has improved, the mortality rate of pulmonary cancer patients remains high. To reduce these high rates of mortality, many researchers have focused on methods for tumor prevention as well as earlier detection and more effective treatments [1]. In recent years, there has been an increasing interest in the search for antioxidative, antiinflammatory, cancer preventative, and therapeutic agents from a wide variety of traditional herbal plants. Recent studies have demonstrated that the consumption of natural foods could eliminate the risk of certain types of human cancers [2, 3]. Many drugs used in the clinical treatment of cancer patients are derived from natural plant species [4, 5].

Turmeric (*Curcuma longa*), a plant native to South and Southeast Asia, has been routinely used in cooking and as a

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Abbreviations: AHR, airway hyperresponsiveness; **IHC,** immunohistochemistry staining; **MMP,** matrix metalloproteinase; **VEGF,** vascular endothelial growth factor

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medicine for thousands of years. Curcumin (diferuloylmethane) is a major active polyphenol compound of turmeric and exhibits remarkable effects on cancer, inflammation, ulcers, wounds, and aging; it additionally provides antioxidative, immunomodulatory, and neuroprotective benefits [6]. Chemopreventive and cell growth inhibitory activities of curcumin have been demonstrated in brain [7], colon [8], bladder [9], breast [10], prostate [11], and cervical [12] cancers. To the best of our knowledge, there have been only a few studies on the inhibition of pulmonary cancer with curcumin, and these studies have relied on an in vitro assay [13–17]. However, at this juncture, we have no definitive information that turmeric blocks pulmonary tumor formation in vivo.

In this study, we investigated the inhibition of pulmonary tumor formation through treatment with curcumin in transgenic mice for the first time. Pulmonary functions were measured by airway hyperresponsiveness (AHR), lung pathological histology, and human vascular endothelial growth factor (hVEGF) immunohistochemistry (IHC) staining during the 5-month treatment with curcumin. The mRNA and protein levels of markers of tumorigenesis and cell cycle regulators were examined by quantitative RT-PCR and Western blots to evaluate the potential for curcumin to be further developed and applied as a clinical anti-pulmonary cancer drug.

2 Materials and methods

2.1 Transgenic mouse production and validation

The mccsp-Vegf-A_{165}-sv40 transgenic mice were generated by pronuclear microinjection. To detect the hVEGF-A_{165} transgene in the transgenic mice with a homozygous (hVEGF-$A_{165}^{+/+}$) or hemizygous (hVEGF-$A_{165}^{+/-}$) genotype, the mice were rapidly screened for the foreign gene by PCR analysis of tail genomic DNA with the primer set VEGF94(+): 5′-AAGGAGGAGGGCAGAATCATC-3′ and VEGF315(−): 5′-GAGGTTTGATCC GCATAATCTG-3′.

2.2 Animals

The transgenic mice were given a standard laboratory diet and distilled water ad libitum and kept on a 12-h light/dark cycle at $22 \pm 2^{\circ}C$. This study was conducted according to institutional guidelines and approved by the Institutional Animal Care and Utilization Committee of National Chung Hsing University, Taiwan (IACUC-98-3). The transgenic mice with the hemizygous (hVEGF-$A_{165}^{+/-}$) genotype were randomly assigned to two groups for treatment ($n = 6$): Tg/Placebo (transgenic mice) and Tg/Curcumin (transgenic mice treated with curcumin) groups. Curcumin (Fig. 1A) obtained from Sigma Chemical (St. Louis, MO, USA) was dissolved in DMSO and injected intraperitoneally (i.p.) at 5 mg/kg body weight (BW) three times a week. Mice were sacrificed when 11 months old, following 5 months of curcumin administration (Fig. 1B). Pulmonary tissues were collected for pathological histology, IHC staining, and extracting RNA and protein according to our previous protocols [18–20].

2.3 Measurement of AHR

To evaluate the AHR in the Tg/Placebo and Tg/Curcumin groups, bronchial provocation tests were performed using

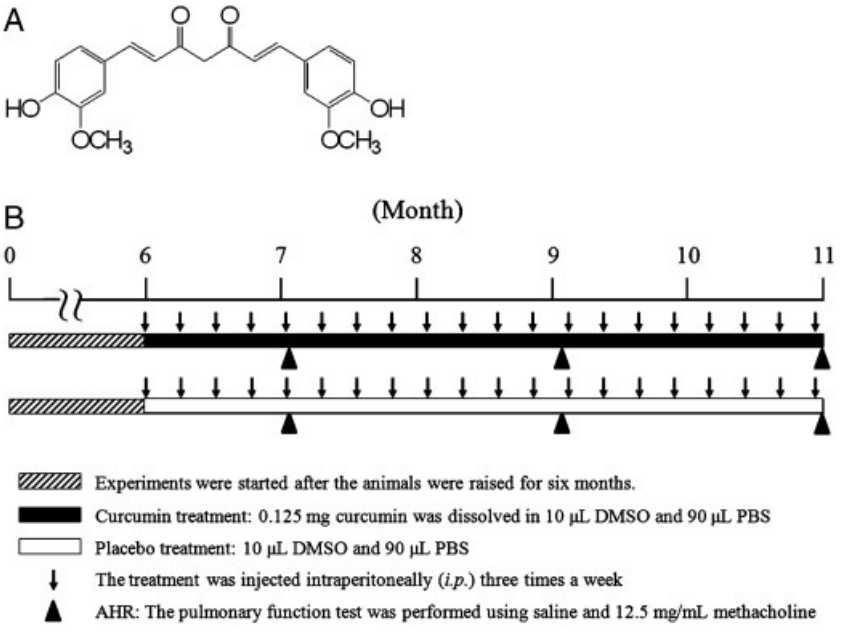

Figure 1. (A) Chemical structures of curcumin. (B) The schedule of curcumin treatment. Curcumin was injected intraperitoneally (i.p.) at 5 mg/kg BW three times a week. Mice were sacrificed when 11 months old after curcumin was administered for 5 months.

methacholine. First, the basal pulmonary function was measured; then, saline and methacholine at a concentration of 12.5 mg/mL were converted to aerosol using a nebulizer and allowed to be inhaled five times through a Rosenthal-French dosimeter. Three minutes later, a pulmonary function test was performed. The pulmonary function of each step was measured 30 times with a portable microspirometer, and the enhanced pause (penh) values were selected as the pulmonary function value.

2.4 Pathological histology

Pulmonary tissue was fixed in 10% buffered formaldehyde and examined using hematoxylin and eosin (H&E) staining as described previously [21, 22].

2.5 IHC staining

Formaldehyde-fixed and paraffin-embedded sections were cut to a thickness of 5 μm, dewaxed and rehydrated through a gradient of alcohols to water, and then treated with boiling water for 15 min. The sections were incubated in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity and then incubated overnight at 4°C with primary rabbit monoclonal antibody against hVEGF-A using a 1:40 working dilution. For antigen retrieval, the sections were immunostained with the VECTASTAIN® ABC kit (UNIVERSAL, VECTOR, USA) in accordance with the manufacturer's specifications. Diaminobenzidine (DAB) was used for staining development, and the sections were counterstained with hematoxylin [23]. The negative control consisted of substituting normal serum for primary antibody.

2.6 Real-time RT-PCR

Total RNA from pulmonary tissue was extracted from the lung tissue using the Trizol reagent (Invitrogen) as specified by the manufacturer. Total RNA (2 μg) was resuspended in 9 μL of diethylpyrocarbonate (DEPC)-treated water, and the first strand of cDNA was synthesized with random primers and ImProm-II™ reverse transcriptase in a total volume of 20 μL. The reaction was carried out at 42°C for 1 h. For further PCR amplification, an aliquot (1:10) of the RT product was adjusted to contain 0.1 μg of each primer, and additional buffer was added to a total volume of 20 μL. RT-PCR was performed in a Thermal Cycler 2720. Real-time RT-PCR was performed using SYBR Green in a Rotor-Gene™ 6000. To evaluate gene expression, real-time RT-PCR was performed on 14 genes (*vegf, kdr, nrp-1, myc, brca-1, mmp2, mmp9, egfr, erk2, survivin, cyclin a, cyclin b1, cyclin d,* and *cyclin e*) using cDNA from pulmonary tissue. The cDNA of *β-actin* was used as an internal control.

2.7 Western blot analysis

Expression of pulmonary tissue protein was measured by Western blot. Pulmonary tissues were homogenized in 500 μL of RIPA buffer (5 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% NP40, 0.25% sodium deoxycholate, 5 mM EDTA, and 1 mM ethylene glycol-bis(2-aminoethyl-ether)-*N,N,N,N*-tetraacetic acid). The homogenates were centrifuged at 12 000 × *g* for 30 min at 4°C. Protein (40 μg) was then separated by SDS-PAGE in 10% polyacrylamide and electrotransferred to polyvinylidene difluoride membranes. The membranes were incubated in blocking solution (5% BSA) at room temperature for 2 h. The membranes were then incubated with primary antibody (VEGF-A, EGFR, ERK2, Cyclin A, Cyclin B, and GAPDH) overnight at 4°C. After washing, the membranes were incubated with a goat anti-rabbit IgG peroxidase-conjugated secondary antibody directed against the primary antibody. The membranes were developed by an enhanced chemiluminescence Western blot detection system as described previously [24].

2.8 Statistical analysis

Experimental values are expressed as the mean ± standard error of mean (SEM). All data were analyzed using the *t*-test. Statistical significances are presented as $p<0.05$ (*) or $p<0.01$ (**).

3 Results

3.1 Effects of curcumin on AHR parameters of pulmonary function

There are several invasive and non-invasive methods that have been used for the investigation of airway responsive-

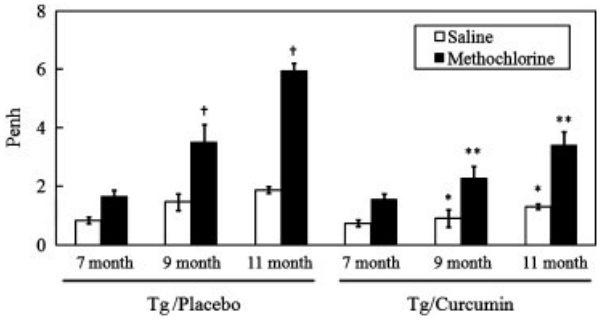

Figure 2. Effects of curcumin on airway hyperresponsiveness (AHR) parameters of pulmonary function. The Penh (enhanced pause) is a dimensionless value that represents a function of the proportion of maximal expiratory to maximal inspiratory box pressure signals and of the timing of expiration. Mean ± SEM ($n=6$). †$p<0.01$ Tg/Placebo versus Tg/Placebo group at 7 months. *$p<0.05$ Tg/Curcumin group versus Tg/Placebo group at the same month. **$p<0.01$ Tg/Curcumin group versus Tg/Placebo group at the same month.

ness in mice in vivo. An invasive method for the detection of pulmonary function involves tracheotomized, endotracheally intubated rodents or orotracheally intubated rodents. Unrestrained barometric plethysmography in conscious mice represents the extreme of non-invasiveness and has been widely used recently for measuring AHR in mice. It is a vital method because of its ability to obtain measurements easily and quickly. AHR has been an important characteristic of pulmonary function, and hyperresponsiveness to methacholine could be both the basic information needed for diagnosis and the determination of the severity of disease. It may also be used as a marker to predict the subsequent development of respiratory diseases. AHR was assessed as a percent increase of Penh in response to increasing damage to pulmonary function. In this study, AHR was measured at 7, 9, and 11 months treated with curcumin (Fig. 2). The penh values for the Tg group treated with methacholine were dramatically elevated to 3.52 at 9 months and 5.94 at 11 months from the starting value of 1.65 at 7 months. However, the group treated with 5 mg/kg of curcumin showed a significant decrease in penh elevation, with values of 1.29 at 7 months, 1.89 at 9 months and 2.02 at 11 months. The result demonstrated that 5 mg/kg of curcumin could effectively reduce pulmonary function damage.

3.2 Effect of curcumin on pathological histology

Figure 3 shows that pulmonary tumors were found in the transgenic mice and primarily included neoplasm growing on the peripheral of the pulmonary alveolus and adenomas growing on the site near the lung bronchus (Fig. 3A and B). In the pulmonary alveolus on the lung bronchus of transgenic mice, some obvious and large-grained pink cells were found. Such pink cells were macrophages and indicative of an inflammation response. These results suggest that hVEGF-A_{165} is capable of promoting vascular permeability and the effectiveness of the inflammation response. Furthermore, treatments with 5 mg/kg of curcumin reduced the neoplasmic growth on the periphery of the pulmonary alveolus and adenomas growing on the site near the lung bronchus (Fig. 3C and D). This histological examination suggests that curcumin has potential anti-cancer and anti-

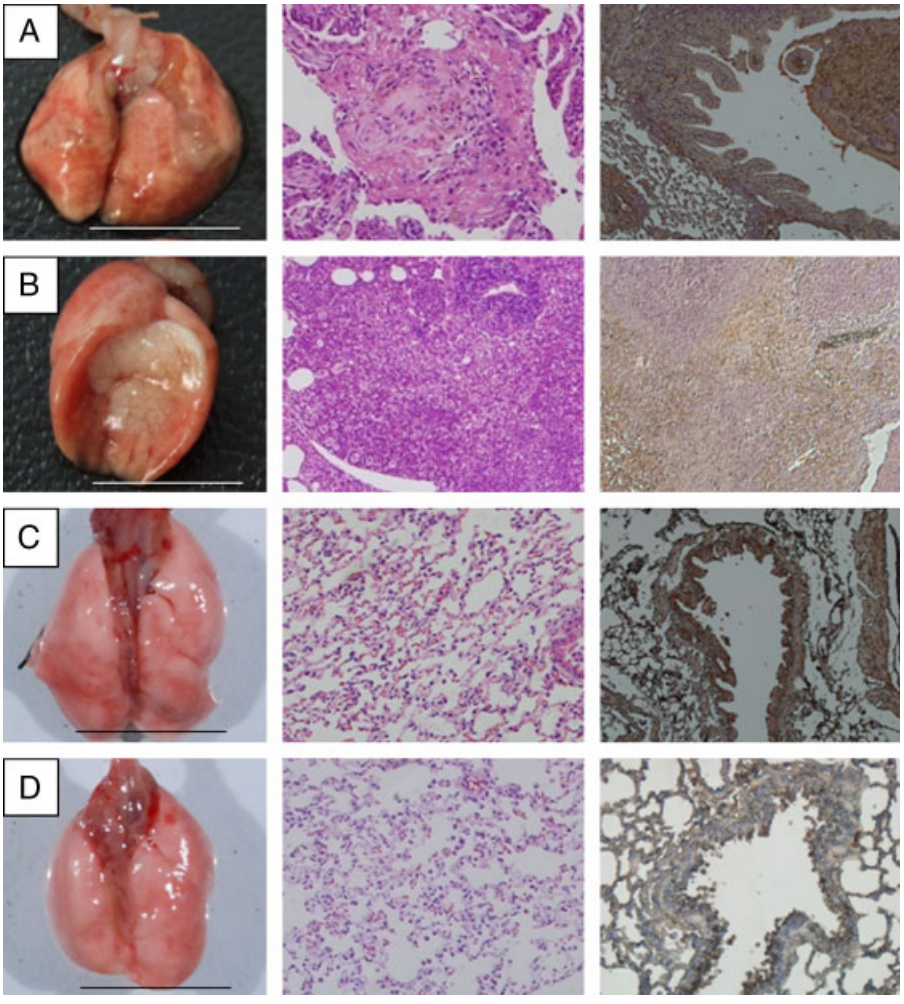

Figure 3. The exterior, histopathological slides and immunohistochemical (IHC) staining of the lung tissues of 11-month-old hVEGF-A_{165}-overexpressing transgenic mice in the (A) Tg/Placebo group, (B) Tg/Placebo group, (C) Tg/Curcumin group, and (D) Tg/Curcumin group after curcumin was administered for 5 months. Scale bar = 1 cm

inflammatory effects in the lungs of hVEGF-A_{165}-overexpressing transgenic mice.

Angiogenesis is an important factor in the formation of tumors as well as in tumor growth, invasion, and metastasis. VEGF promotes the initial formation of new blood vessels (vasculogenesis) and plays a vital role in the growth and expansion of these new blood vessels (angiogenesis). Its role in tumor growth is basic because a tumor cannot exceed 1–2 mm in diameter or thickness without vascularization [25]. In addition, angiogenesis is important to the formation of metastases. In this study, *ccsp-Vegf-A165-sv40 poly(A)* was constructed for the production of lung-specific overexpressing VEGF-A_{165} transgenic mice by microinjection, and thus the hVEGF-A_{165} is specific expression in Clara cells of lung tissues. In the IHC staining of Fig. 3A and B, we found that not only hVEGF-A_{165} was overexpressed in Clara cells of lung tissues but also endogeneous VEGF was overexpressed in lung cells except for Clara cells (both of human and mouse VEGF can be detected by this VEGF antibody). Furthermore, the treatment with curcumin significantly blocked both transgen-hVEGF and endogeneous VEGF overexpression in lung tissues (Fig. 3C and D). These results indicated that transgenic mice treated with curcumin can reduce both transgen-hVEGF and endogeneous VEGF overexpression compared with untreated transgenic mice, which in turn could eliminate the formation and growth of new blood vessels. The anti-vasculogenic and anti-angiogenic properties of curcumin may thus represent a possible cause for the previously observed suppression of tumor growth, invasion, and metastasis.

3.3 Curcumin causes the suppression of marker genes for tumor formation

It is expected due to its previously shown association with the formation of lung cancer that curcumin could inhibit expression of genes responsible for cell proliferation and survival. The mRNA expression patterns of *vegf*, *kdr*, *nrp-1*, *myc*, *brca-1*, *mmp2*, *mmp9*, *egfr*, *erk2*, *survivin*, *cyclin a*, *cyclin b1*, *cyclin d*, and *cyclin e* in the Tg/Placebo and Tg/Curcumin groups were assessed using Q-PCR (Fig. 4). Previous studies have reported that curcumin suppresses the matrix metalloproteinase family (MMPs), especially MMP-2 and MMP-9, which are believed to be involved in tumor angiogenesis due to their matrix degrading capacity [26–28]. We showed that curcumin slightly reduced the expression of mmp-2 and mmp-9, but the difference was not statistically significant. In addition, curcumin treatment of Tg mice markedly decreased the mRNA levels of *vegf*, *kdr*, *nrp-1*, *c-myc*, *egfr*, *erk2*, and *cyclin a*. *Kdr* and its co-receptor *nrp-1* are the main angiogenic receptors in the vegf pathway. We observed that curcumin reduces mRNA expression levels of kdr and its co-receptor nrp-1. It is possible that inhibition of signaling downstream of *kdr* reduces the expression of the VEGF. Furthermore, we found that *erk2* downregulation was followed by a downstream reduction of *c-myc* expression, which may contribute to the aforementioned G1 arrest. Additionally, *cyclin a* is critical for *c-myc*-modulated cell progression.

3.4 Curcumin causes suppression of cell cycle progression signaling pathways

Western blotting showed that ERK2, Cyclin A, and Cyclin B were downregulated in Tg mice following curcumin treatment (Fig. 5). A possible reason for this cell cycle effect is the decrease in protein levels of Cyclin A and Cyclin B. Cyclin A is accumulated in the S phase of the cell cycle and degraded at the late S/G2 phase [29], and cyclin B is accumulated at the late S/G2 phase and degraded in the M phase [30]. Treatment with curcumin would affect the degradation of *cyclin a* at the late S/G2 phase, without significant effect on *cyclin b* in the M phase. Furthermore, we found that Cyclin B protein levels were significantly reduced by curcumin. Decreased Cyclin B synthesis can be due to the

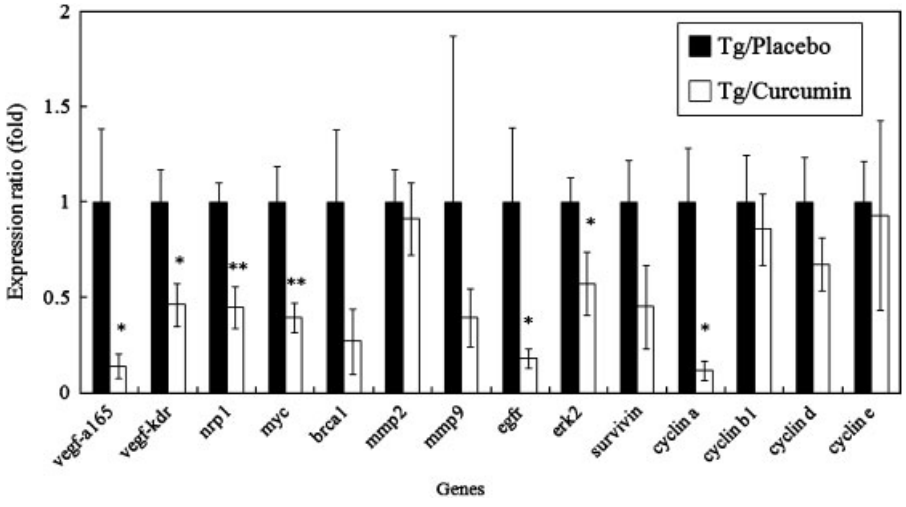

Figure 4. Real time-PCR validations of mRNA expression levels of *vegf*, *kdr*, *nrp-1*, *myc*, *brca-1*, *mmp2*, *mmp9*, *egfr*, *erk2*, *survivin*, *cyclin a*, *cyclin b1*, *cyclin d*, and *cyclin e* in the lung tissues of the Tg/Placebo group and Tg/Curcumin group. β-actin was used as an internal control. The quantitative mRNA expression levels were measured by Q-PCR. Mean ± SEM ($n = 6$). $^{*}p < 0.05$ versus Tg/Placebo group. $^{**}p < 0.01$ versus Tg/Placebo group.

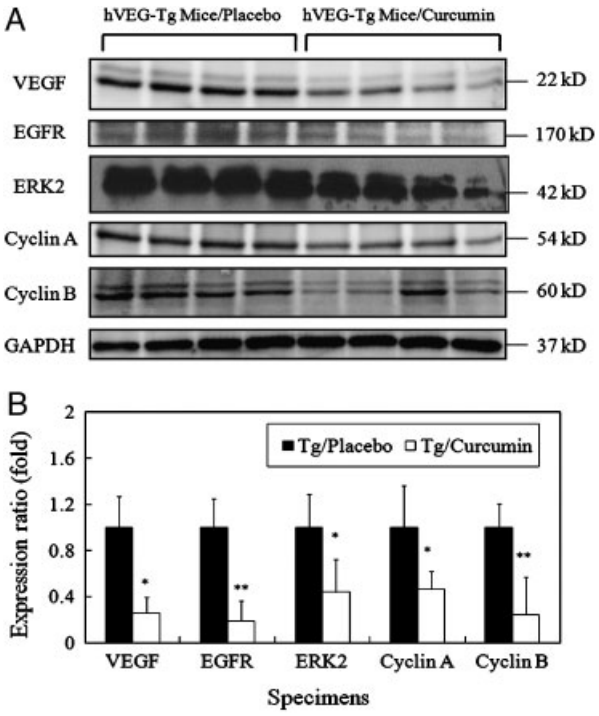

Figure 5. Protein expression levels of VEGF-A, EGFR, ERK2, Cyclin A, and Cyclin B in the lung tissues of the Tg group and Tg/curcumin group as measured by Western blot. GAPDH was used an internal control. Mean ± SEM ($n = 6$). $^{*}p < 0.05$ versus Tg group. $^{**}p < 0.01$ versus Tg group.

degradation of *cyclin b* mRNA or inhibition of *cyclin b* mRNA synthesis. However, we found *cyclin b* mRNA synthesis is not inhibited. It is possible that the translate effect of *cyclin b* is reduced or the stability of the protein of Cyclin B is reduced, which leads to the degradation of Cyclin B protein. These results indicate that the protein expressions involved in the S to M phase transition in Tg mice were suppressed by curcumin.

4 Discussion

Previous studies have shown that curcumin, the active constituent of turmeric, possesses potent antioxidant, anti-inflammatory, and anti-cancer properties [6]. Curcumin has long been known as a drug or preventive agent for cancers that regulates a variety of antitumor signaling pathways [31]. However, until now, we had no definitive information that curcumin blocks pulmonary tumor formation in vivo. Therefore, in this study, we investigated the inhibition of pulmonary tumor formation by curcumin in transgenic mice for the first time.

A major source of human consumption of curcumin is from turmeric, which is used extensively in curry and mustard and as a coloring agent and spice in many foods. It has been estimated that some individuals ingest as much as 600 mg of dietary turmeric (10–30 mg of curcumin) in their diet daily [32]. Thus, in this study we simulate the diet of curcumin to treat the mice (5 mg/kg BW of mice = 0.5549 mg/kg of human = 33.294 mg/60 kg of human). Our previous study provided a method for producing an animal model for pulmonary cancers to study the regulatory mechanism of pulmonary adenocarcinoma. Thus, we investigated the anti-lung cancer effect of curcumin using an 11-month-old lung-specific hVEGF-A_{165}-overexpressing transgenic mice model that constructed m*ccsp-Vegf-*A_{165}*-sv40 poly(A)* transgene. We found that following a 5-month treatment with curcumin, 11-month-old hVEGF-A_{165}-overexpressing transgenic mice showed a dramatic decrease in solid tumor formation compared to untreated transgenic mice. Histological examination showed that treatment with 5 mg/kg of curcumin reduced pulmonary tumor formation and inflammation in hVEGF-A_{165}-overexpressing transgenic mice (Fig. 3). In this study, we found that curcumin reduced VEGF overexpression, which is related to vasculogenesis and angiogenesis and is a vital factor in the formation of tumors as well as tumor growth, invasion, and metastasis. Furthermore, El-Azab et al. [33] showed that treatment with curcumin significantly reduced plasma levels of endogeneous VEGF overexpression in Ehrlich ascites tumor-bearing mice. Aggarwal et al. [34] pointed out curcumin suppressed the paclitaxel-induced expression of VEGF in breast cancer cells. Our finding is in agreement with the results reported by El-Azab et al. [33] and Aggarwal et al. [34], claiming that curcumin could significantly suppress the expression of transgen-hVEGF and endogeneous VEGF.

Recently, there has been an extensive evaluation of the usage plants and their phytochemicals in the treatment of cancers. In particular, the active phytochemicals that function as cell cycle modulators are gaining widespread attention [35]. In this study, our results suggest that curcumin can eliminate the damage to pulmonary function (Fig. 2) and the formation of pulmonary cancer (Fig. 3) through the VEGF, EGFR, ERK2, Cyclin A, and Cyclin B proteins (Fig. 5). Furthermore, our findings are also in agreement with the results reported by Wu et al. [15], claiming that treatment with curcumin significantly reduced Cyclin A expression in a dose-dependent manner in human non-small cell lung NCI-H460 cells. Curcumin has also been shown to suppress the proliferation of human colon and bladder cancer cells in culture through the downregulation of cyclin A and upregulation of p21 [36, 37]. Moreover, Kim and Lee [38] showed that curcumin-treated cancer cells, expression of E2F4 itself and several E2F4 downstream genes, E2F1, c-myc, CDK2, cyclin A, cyclin D1, and p27, which are involved in cell cycle control and related to the apoptotic cell death was reduced. Furthermore, Qin et al. [39] also found that E2F, another key regulator for G1 to S phase, is also targeted by curcumin. In addition, in this study, we found that curcumin reduced the expression of cyclin A. Therefore, treatment with curcumin could affect the expression of E2F, which therefore leads to

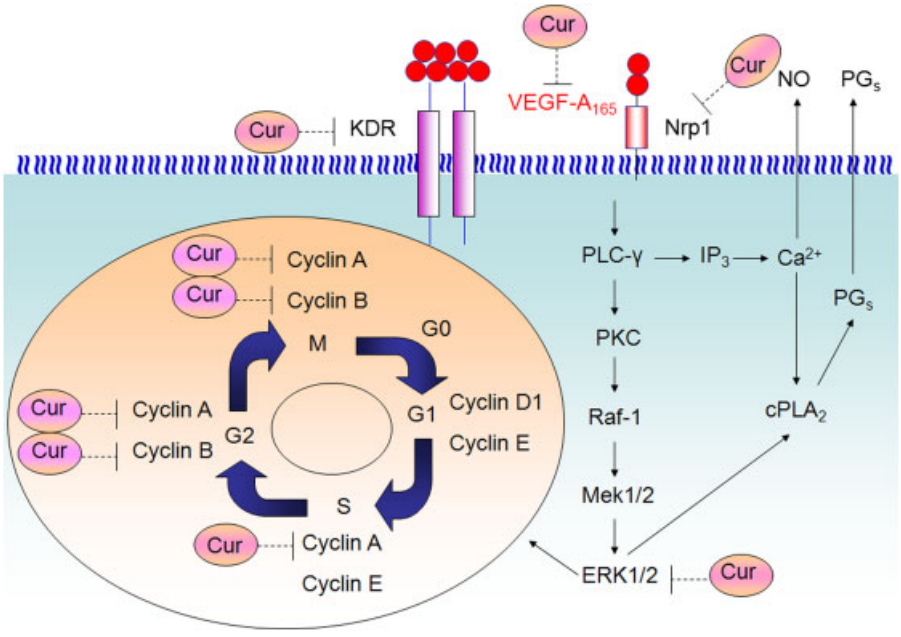

Figure 6. Scheme of the curcumin regulatory pathway. The effects of curcumin on lung tumor formation are hypothesized to occur through the cell cycle signaling pathway. The diagram shows that curcumin might inhibit the expressions of VEGF-A, KDR, Nrp1, ERK2, Cyclin A, and Cyclin B.

downregulation of cyclin A. Additionally, we have found that treatment with curcumin in 11-month-old hVEGF-A_{165}-overexpressing transgenic mice dramatically decreases tumor formation compared with untreated transgenic mice. These results suggest that a blockade of the cell cycle may be the critical mechanism for the observed effects following treatment with curcumin. It is also possible that the inhibition of signaling downstream of EGFR reduces the expression of VEGF through an indirect mechanism. Here, we show a relationship between curcumin treatment and the expression of VEGF, EGFR, ERK2, and Cyclin A at the transcriptional and translation levels (Fig. 6).

In conclusion, curcumin is an antioxidant and anti-inflammatory compound that possesses a variety of pharmacological functions. Thus, the combination of all of curcumin's pharmacological functions may be needed to explain its role in the treatment of various cancers. Though several studies have demonstrated the potential role of curcumin in animals, further studies in humans are required to confirm these results.

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The authors have declared no conflict of interest.

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