High Dietary Inorganic Phosphate Increases Lung Tumorigenesis and Alters Akt Signaling

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***Rationale*: Phosphate (Pi) is an essential nutrient to living organisms. Recent surveys indicate that the intake of Pi has increased steadily. Our previous studies have indicated that elevated Pi activates the Akt signaling pathway. An increased knowledge of the response of lung cancer tissue to high dietary Pi may provide an important link between diet and lung tumorigenesis.**

***Objectives*: The current study was performed to elucidate the potential effects of high dietary Pi on lung cancer development.**

***Methods*: Experiments were performed on 5-week-old male K-*ras*^{LA1} lung cancer model mice and 6-week-old male urethane-induced lung cancer model mice. Mice were fed a diet containing 0.5% Pi (normal Pi) and 1.0% Pi (high Pi) for 4 weeks. At the end of the experiment, all mice were killed. Lung cancer development was evaluated by diverse methods.**

***Measurement and Main Results*: A diet high in Pi increased lung tumor progression and growth compared with normal diet. High dietary Pi increased the sodium-dependent inorganic phosphate transporter-2b protein levels in the lungs. High dietary consumption of Pi stimulated pulmonary Akt activity while suppressing the protein levels of tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 as well as Akt binding partner carboxyl-terminal modulator protein, resulting in facilitated cap-dependent protein translation. In addition, high dietary Pi significantly stimulated cell proliferation in the lungs of K-*ras*^{LA1} mice.**

***Conclusions*: Our results showed that high dietary Pi promoted tumorigenesis and altered Akt signaling, thus suggesting that careful regulation of dietary Pi may be critical for lung cancer prevention as well as treatment.**

Keywords: inorganic phosphate; lung tumorigenesis; Akt signaling

Inorganic phosphate (Pi) is present in bacterial, fungal, plant, and animal cells. Pi plays a critical role in diverse cellular functions involving intermediary metabolism and energy-transfer mechanisms. It is a vital component of phospholipids in membranes and of nucleotides, both of which provide energy and serve as components of DNA, RNA, and phosphorylated intermediates in cellular signaling (1). Pi balance is regulated by the family members of sodium-dependent inorganic phosphate transporters (NPT) that regulate entrance into the cellular membrane (2). NPTs are subdivided into three families based, in part, on tissue specificity (3). These families differ by their affinity for Pi, distribution in the body, and by the mechanisms that control their activity (4, 5). Among them, the NPT-2b is expressed at the apical domain of enterocytes in the small intestine, lung, testis, and mammary gland (6, 7). In addition, the expression of NPT-2b was increased by dietary Pi in the lungs of mice (8), and NPT-2b is a potential target for cancer therapy (9).

Lung cancer is currently the most frequently diagnosed solid tumor in the world and is the most common cause of cancer mortality worldwide. Non–small cell lung cancer (NSCLC) constitutes over 75% of lung cancers and has an average overall 5-year survival rate of 14% (10). Reports have indicated that approximately 90% of NSCLC was associated with constitutive activation of the phosphatidylinositol 3′-kinase (PI3K)/Akt pathway, and that such Akt activation promoted cellular survival and resistance to chemotherapy or γ-irradiation (11). Akt/protein kinase B is serine/threonine kinase, which is activated in cells exposed to diverse stimuli such as hormones, growth factors, and extracellular matrix components. Such Akt also has emerged as a crucial regulator of widely divergent cellular processes including proliferation, differentiation, and metabolism (12).

Phosphate is being added to a large number of processed foods because the addition of Pi increases the quality of food through improved water retention and texture (13). Surveys conducted in various countries indicate that intake of Pi has increased steadily as Pi-containing foods increased by approximately 17% in the decade leading up to 1993 (14). These surveys also suggest that the

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The Akt pathway plays an important role in the lung tumorigenesis, and recent advances suggest that elevated Pi may activate the Akt signaling in the normal lungs.

What This Study Adds to the Field

High dietary inorganic phosphate strongly activates Akt signaling, and increased lung tumorigenesis. Careful regulation of dietary Pi may be critical for lung cancer prevention as well as treatment.

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use of Pi as a food additive may continue to increase (14). Our previous studies found that high dietary Pi might activate the Akt pathway in the lungs of mice (8), and the activation of Akt signaling was dependent on Pi treatment in normal lung cells (15). *In vitro* studies indicate that Pi works as a stimulus capable of increasing or decreasing the expression of a number of pivotal genes such as those involved in the regulation of transcription, signal transduction, and cell cycle (15–20). It is thought that excess amount of Pi intake over a long period of time is a strong factor in aging (1). Therefore, regulating appropriate Pi consumption seems to be important in maintaining a high quality of life. However, studies have not yet investigated homeostatic maintenance of the tumorigenic lung's responses to an increased uptake of dietary Pi.

The current study was performed to elucidate the potential effects of high Pi levels on lung cancer development using K-*ras*^{LA1} mice, a laboratory animal model of lung cancer. Moreover, lung tumors in the mice have morphologic, histogenic, and molecular features similar to human lung adenocarcinoma (21, 22). In addition, K-*ras* is the most frequently mutated member in human tumors, including adenocarcinomas of the lungs (~25–50%) (21). In K-*ras*^{LA1} mice, oncogenic alleles of K-*ras* are activated by a spontaneous recombination event resulting in tumorigenesis with the most frequent organ site being the lung (23). These mice develop varying grades of tumors from hyperplasia to carcinomas, similar to human NSCLC (23). We also used a second model, the urethane-induced lung cancer model mice. Approximately 80% of the urethane-induced lung tumors harbor a mutation in the K-*ras* gene, making this another frequently used and valuable lung cancer model (24, 25).

The results of this study revealed that increased uptake of dietary Pi stimulated pulmonary tumorigenesis and altered Akt signaling as well as cap-dependent protein translation and cell

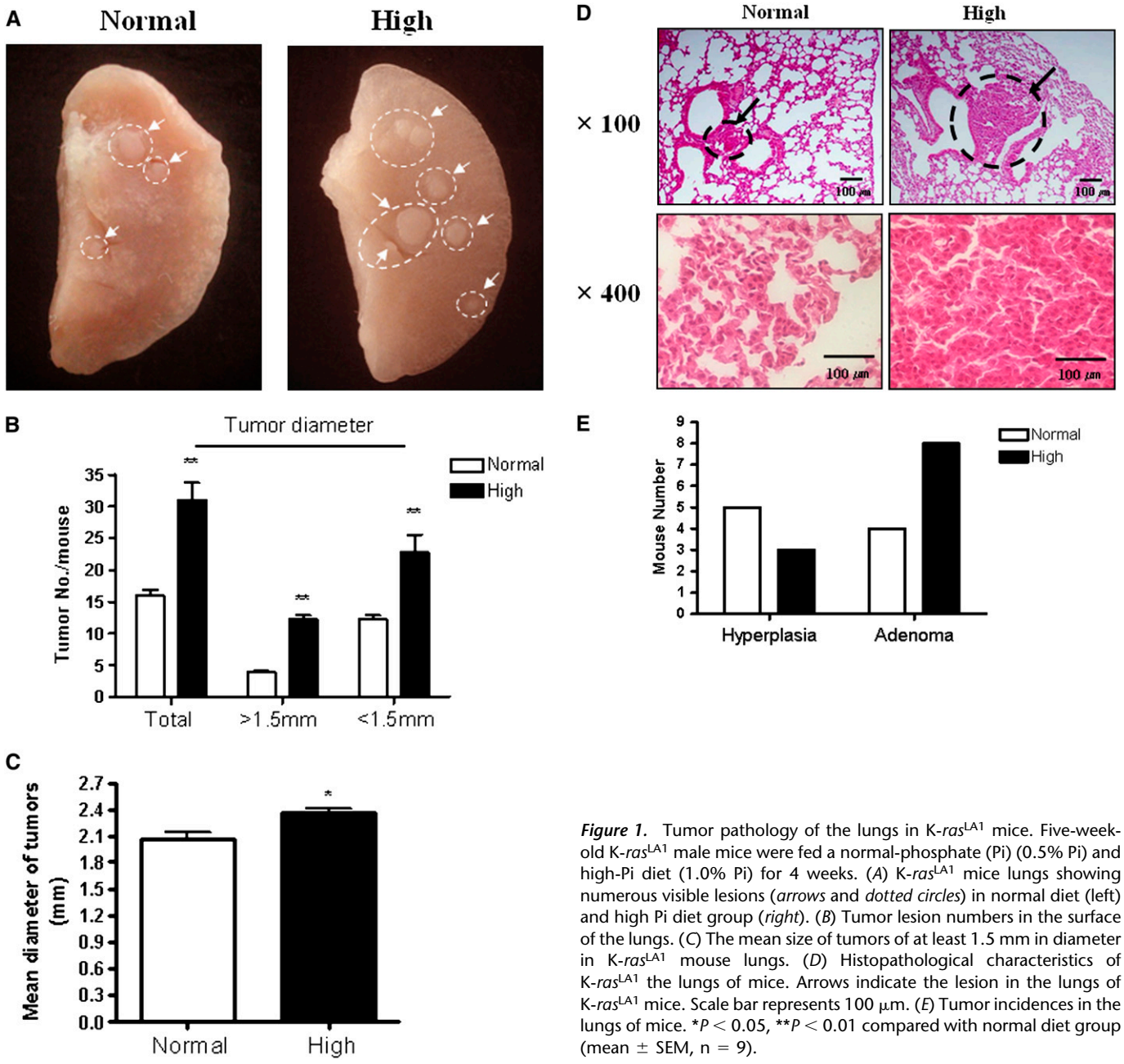

Figure 1. Tumor pathology of the lungs in K-*ras*^{LA1} mice. Five-week-old K-*ras*^{LA1} male mice were fed a normal-phosphate (Pi) (0.5% Pi) and high-Pi diet (1.0% Pi) for 4 weeks. (*A*) K-*ras*^{LA1} mice lungs showing numerous visible lesions (*arrows* and *dotted circles*) in normal diet (left) and high Pi diet group (*right*). (*B*) Tumor lesion numbers in the surface of the lungs. (*C*) The mean size of tumors of at least 1.5 mm in diameter in K-*ras*^{LA1} mouse lungs. (*D*) Histopathological characteristics of K-*ras*^{LA1} the lungs of mice. Arrows indicate the lesion in the lungs of K-*ras*^{LA1} mice. Scale bar represents 100 μm. (*E*) Tumor incidences in the lungs of mice. $*P < 0.05$, $**P < 0.01$ compared with normal diet group (mean ± SEM, n = 9).

cycle. Our results support the hypothesis that Pi works as a stimulus capable of increasing or decreasing several pivotal genes for lung cancer growth and suggest that regulation of Pi consumption may be important for lung cancer prevention as well as treatment.

METHODS

Animals and Diet

Experiments were performed on 5-week-old male K-*ras*^{LA1} mice and 6-week-old male A/J mice, respectively. Animals were kept in the laboratory animal facility with temperature and relative humidity maintained at 23 ± 2% and 50 ± 20%, respectively, under a 12-hour light/dark cycle. All methods used in this study were approved by the Animal Care and Use Committee at Seoul National University (SNU-060804–4). Breeding K-*ras*^{LA1} mice were obtained from the Human Cancer Consortium – National Cancer Institute (Frederick, MD) and the 18 K-*ras*^{LA1} mice randomly allocated to two groups (9 mice/group); one group received an AIN93-based diet containing 0.5% Pi (normal Pi) and the other group received the same diet fortified with 1.0% Pi (high Pi). All diets were prepared according to the guidelines of the American Institute of Nutrition (AIN) thus fulfilling the requirement for normal growth described precisely by Reeves and colleagues (26). The mice were on the specified diet for 4 weeks. At the end of the experiment, all mice were killed, and blood was taken by cardiac puncture and the serum was separated. During the autopsy procedure, the lungs were perfused carefully, the tumor lesions of lung surfaces were carefully counted, and the lesion diameter was measured with the aid of digital calipers under a microscope, as described by Singh and colleagues (27). Simultaneously, a lobe of the left lung was fixed in 10% neutral buffered formalin for histopathological examination and immunohistochemistry (IHC). Remaining lobes of the lung were stored at −80°C for further analysis. We also tested the effects of high a Pi diet on lung tumorigenesis using the urethane-induced lung cancer mouse model. A/J male mice, 6 weeks of age, were given a single intraperitoneal injection of urethane (1 mg/g body weight) freshly dissolved in 0.9% saline or of saline only, as described by Kisley and colleagues (28). Four weeks after the urethane injection, the mice were divided into two groups (seven mice/group) and fed for 4 weeks a high Pi and normal diet, respectively.

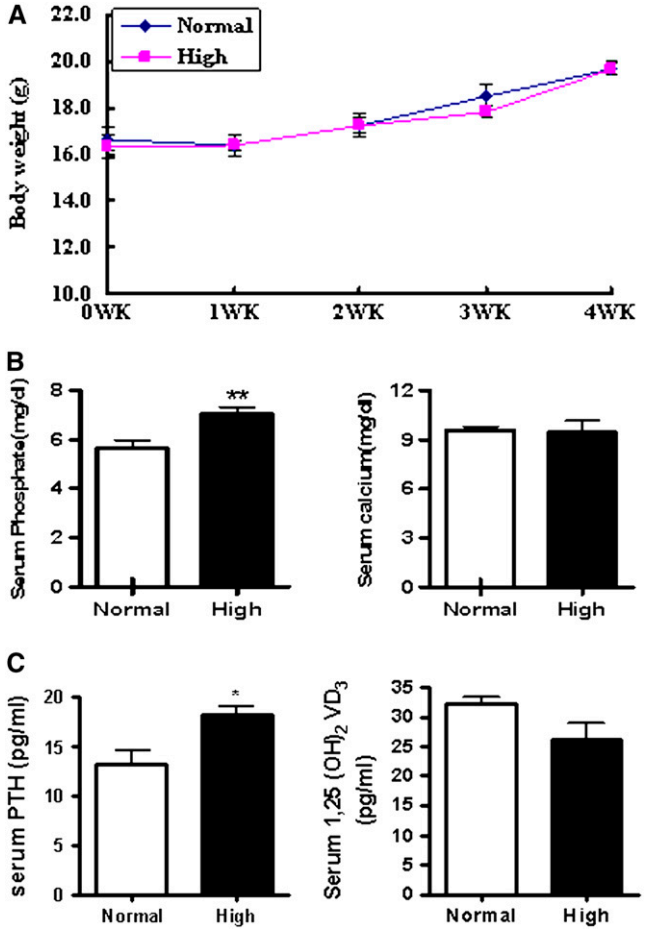

Figure 2. Changes in body weight and serum phosphate (Pi), calcium, serum parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3 (1,25-$[OH]_2$ VD_3) in response to a high Pi diet. Five-week-old K-*ras*^{LA1} male mice were fed a normal (0.5% Pi) and high Pi diet (1.0% Pi) for 4 weeks. (*A*) Changes in body weight were measured once a week for 4 weeks. (*B*) Changes in serum calcium and phosphate levels in response to high and normal phosphate diet. (*C*) Changes in serum parathyroid hormone (PTH) and 1,25 $(OH)_2$ VD_3 levels in response to high and normal phosphate diet. $^{**}P < 0.01$ compared with normal diet group (mean ± SEM, n = 9).

Blood Sample Analysis

The serum levels of Ca^{2+} and Pi were analyzed using a biochemical autoanalyzer (VITALAB, Merck, The Netherlands). Parathyroid hormone (PTH) concentration was measured using an ELISA kit (Immutopics Inc.; San Clemente, CA), and 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2$ VD_3] was measured with radioimmunoassay (RIA) kit (Immunodiagnostic Systems Inc., Fountain Hills, AZ) according to manufacturer's instruction.

Western Blot Analysis

Lung tissues were homogenized with lysis buffer and the protein concentration of the lysate was measured using a Bradford kit (Bio-Rad, Hercules, CA). Equal amounts (30 μg) of protein were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked for 1 hour in Tris-Buffered Saline + Tween 20 containing 5% skim milk, and immunoblotting was performed by incubating the membranes overnight with their corresponding primary antibodies (1:1,000 dilution) at 4°C. Monoclonal antibodies against total Akt1, p-Akt at Ser473, and carboxyl-terminal modulator protein (CTMP) were raised using a general method described elsewhere (29). NPT-2b, p-Akt at Thr308, eukaryotic translation initiation factor 4E (eIF4E),

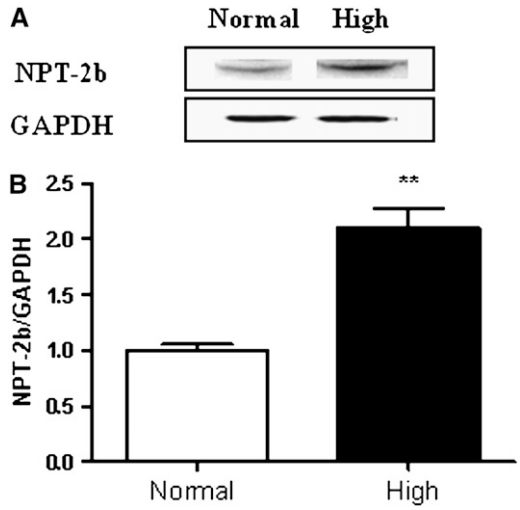

Figure 3. Western blot analysis of sodium-dependent inorganic phosphate transporter (NPT)-2b protein expression in response to high dietary phosphate (Pi) in the lungs of K-*ras*^{LA1} mice. Five-week-old K-*ras*^{LA1} male mice were fed a normal (0.5% Pi) and high Pi diet (1.0% Pi) for 4 weeks. (*A*) Western blot analysis expression of NPT-2b protein in the lungs. (*B*) The bands-of-interest were further analyzed by densitometer. $^{**}P < 0.01$ compared with normal diet group (mea ± SEM, n = 9). GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

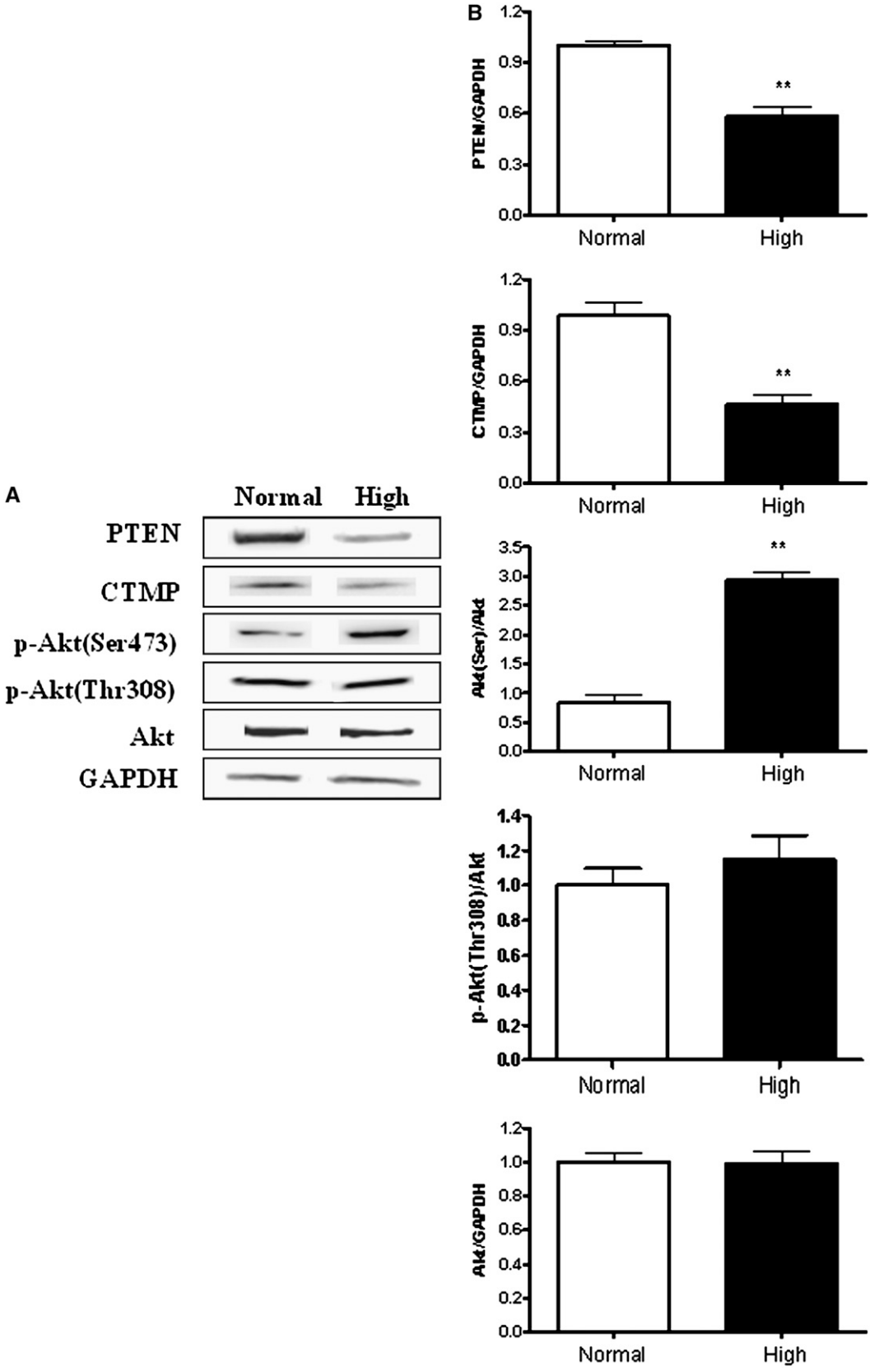

Figure 4. Western blot (WB) analysis of chromosome ten (PTEN), carboxyl-terminal modulator protein (CTMP), Akt and phosphor-Akt (p-Akt) proteins in the lungs of K-*ras*^{LA1} mice. Five-week-old K-*ras*^{LA1} male mice were fed a normal or high phosphate (Pi) diet for 4 weeks. Lung tissue homogenates were subjected to Western blot analysis. Blots were probed with antibodies as indicated. (*A*) Expression of PTEN, CTMP, Akt, p-Akt at Ser473 and p-Akt at Thr308 proteins. (*B*) The bands-of-interest were further analyzed by densitometer. (*C*) Akt kinase activity was measured in the lung homogenates. (*D*) The bands-of-interest were further analyzed by densitometer. *$P < 0.05$, **$P < 0.01$ compared with normal diet group (mean ± SEM, n = 9). GAPDH = glyceraldehyde-3-phosphate dehydrogenase; I.P. = immunoprecipitation; p-GSK 3 = phosphor-glycogen synthase kinase.

eIF4E-binding proteins (4E-BP1), phosphor-4E-BP1 (p-4E-BP1), proliferating cell nuclear antigen (PCNA), cyclin D3 and cyclin dependent kinase 2 (CDK2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mammalian target of rapamycin (mTOR), phosphor-mTOR (p-mTOR), and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) were obtained from Cell Signaling Technology (Beverly, MA). Glyceraldehyde-3-phosphate dehydrogenase antibody was obtained from BD Biotechnology (San Jose, CA). After washing in Tris-Buffered Saline + Tween 20, the membranes were incubated with a horseradish peroxidase-labeled secondary antibody

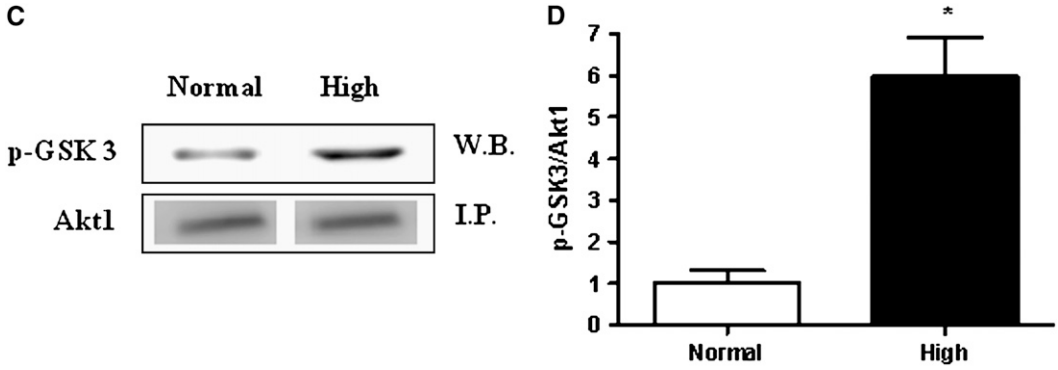

Figure 4. (*continued*)

and the bands of interest were detected using a luminescent image analyzer, LAS-3000 (Fujifilm, Tokyo, Japan). Results were quantified using Multi Gauge software, version 2.02, of the LAS-3000.

Immunoprecipitation and Kinase Assays

Immunoprecipitation of mTOR and eIF4E were performed using Seize primary mammalian immunoprecipitation kit (Pierce, Rockford, IL) and according to manufacturer's instructions. mTOR kinase assay was performed with 300 μmol ATP and 1 μl of phosphorylated heat- and acid-stable protein 1 (PHAS I/4E-BP1; Calbiochem; San Diego, CA) for 30 minutes at 30°C. Reactions were terminated by adding 5X sample buffer and boiling. Samples were analyzed by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Kinase activity of Akt was examined with Akt kinase assay kit (Cell Signaling Technology; Beverly, MA) according to the manufacturer's instruction.

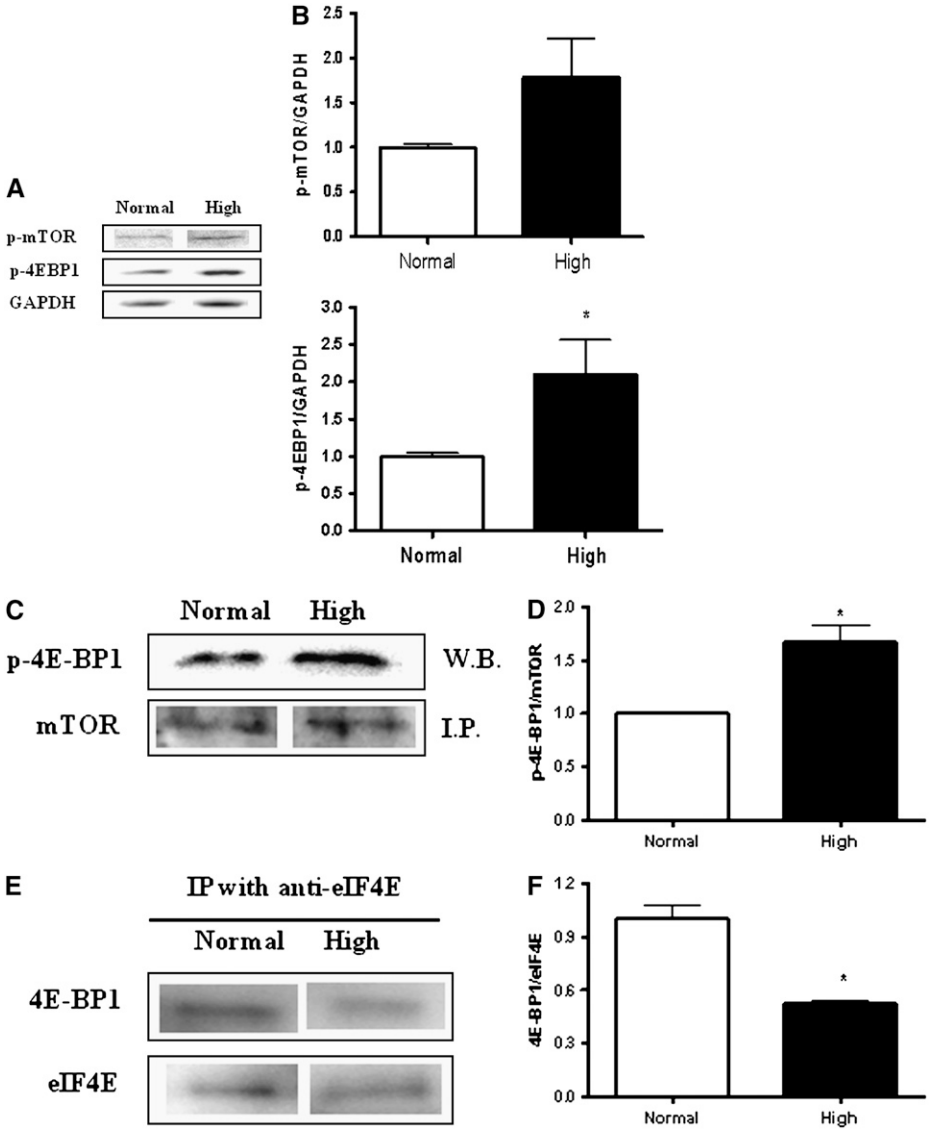

Figure 5. Western blot analysis of Akt downstream signals in the lungs of K-*ras*^{LA1} mice. Five-week-old K-*ras*^{LA1} male mice were fed a normal or high Pi diet for 4 weeks. Lung tissue homogenates were subjected to Western blot analysis. Blots were probed with antibodies as indicated. (*A*) Expression of phosphor-mammalian target of rapamycin (p-mTOR) and p-4E binding proteins (p-4E-BP1) in the lungs. (*B*) The bands-of-interest were further analyzed by densitometer. (*C*) mTOR kinase activity was measured in the lung homogenates. (*D*) The bands-of-interest were further analyzed by densitometer. (*E*) eukaryotic translation initiation factor 4E (eIF4E) immunoprecipitation assay. (*F*) The bands-of-interest were further analyzed by densitometer. **P* < 0.05, ***P* < 0.01 compared with normal diet group (mean ± SEM, n = 9). GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

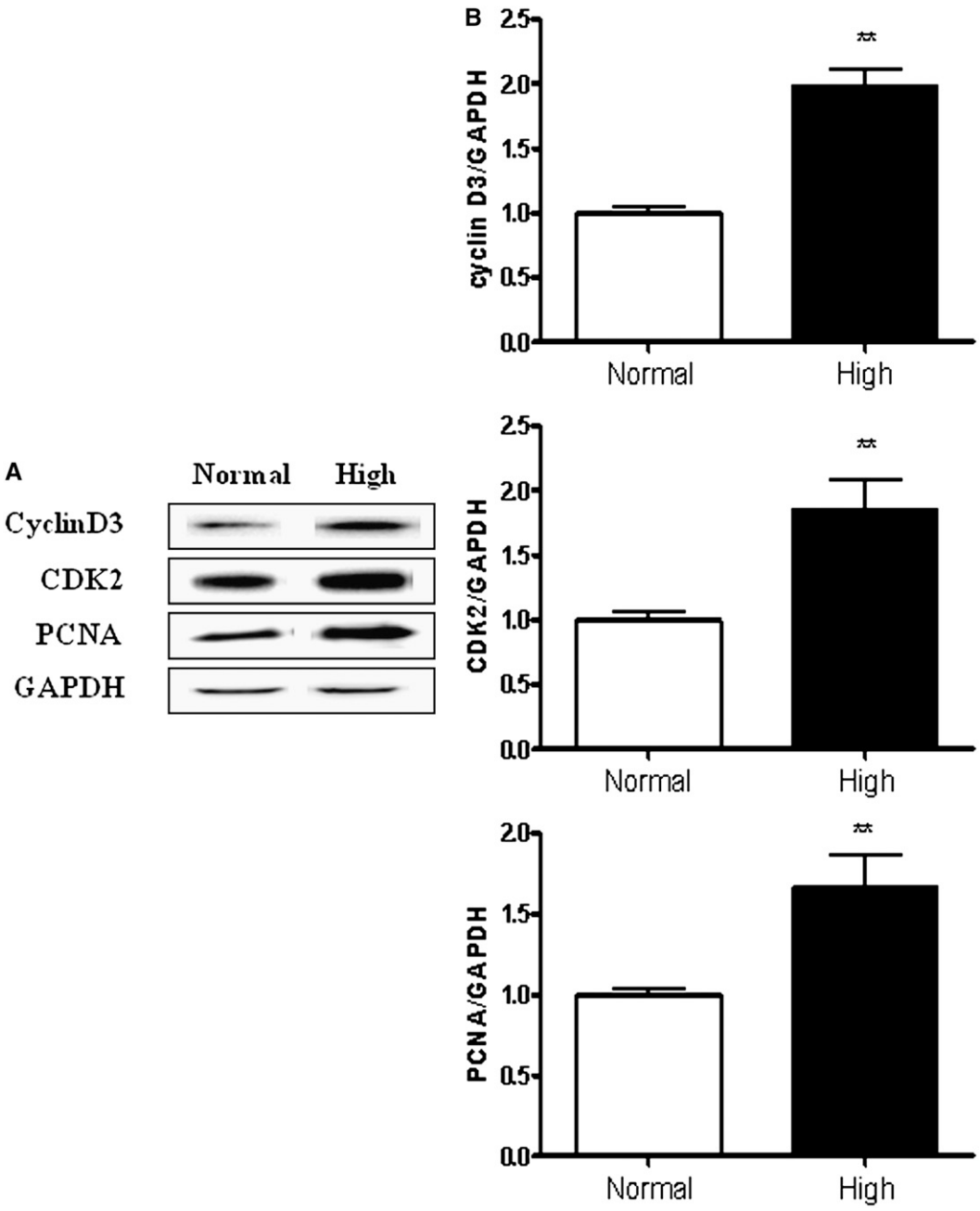

Figure 6. Western blot analysis of proliferating cell nuclear antigen (PCNA), cyclin D3, and cyclin-dependent kinase 2 (CDK2) proteins and immunohistochemistry analysis of PCNA in the lungs of K-*ras*^{LA1} mice. Five-week-old K-*ras*^{LA1} male mice were fed a normal or high phosphate (Pi) diet for 4 weeks. Lung tissue homogenates were subjected to Western blot analysis. Blots were probed with antibodies as indicated. (*A*) Expressions of PCNA, cyclin D3, and CDK2 proteins in the lungs. (*B*) The bands-of-interest were further analyzed by densitometer. (*C*) PCNA immunohistochemistry in the lungs. Dark brown color indicates the PCNA expression (×400; Bar = 50 μm). (*D*) Comparison of PCNA labeling index in lungs of K-*ras*^{LA1} mice. PCNA-positive staining was determined by counting 10 randomly chosen fields per section, determining the percentage of 3,3′-diaminobenzidine-positive cells per 100 cells at ×400 magnification. $*P < 0.05$, $**P < 0.01$ compared with normal diet group (mean ± SEM, n = 9). GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

Histopathological Examination and Immunohistochemistry

The lung tissues were fixed in 10% neutral buffered formalin, paraffin processed, sectioned at 4 μm. For histological analysis, the tissue sections were stained with hematoxylin and eosin. Pulmonary lesions of the mouse were classified using the methods of Nikitin and colleagues (30). For IHC, tissue sections were deparaffinized in xylene and rehydrated through alcohol gradients, then washed and incubated in 3% hydrogen peroxide (AppliChem; Darmstadt, Germany) for 30 minutes to quench endogenous peroxidase activity. After washing in phosphate buffered saline (PBS), the tissue sections were incubated with 5% bovine serum albumin (BSA) in PBS for 1 hour at room temperature to block unspecific binding sites. Primary antibody for PCNA was applied to tissue sections overnight at 4°C. The following day, the tissue sections were washed and incubated with secondary horseradish peroxidase-conjugated antibodies (1:50) for 1 hour at room temperature. After careful washing, tissue sections were counterstained with Mayer's Hematoxylin (Dako; Carpinteria, CA) and washed with xylene. Cover slips were mounted using Permount (Fisher; Pittsburgh, PA), and the slides were reviewed using a light microscope (Carl Zeiss; Thornwood, NY). PCNA positive staining was determined by counting 10 randomly chosen fields per section and determining the percentage of 3,3′-diaminobenzidine-positive cells (nucleus stained cell) per 100 cells at ×400 using the method described by Zhang and colleagues (31).

Statistical Analyses

Quantification of Western blot analysis was performed using Multi Gauge software, version 2.02 (Fujifilm). All results are given as means ± SEM. Results were analyzed by Student's *t* test (Graphpad Software, San Diego, CA). $P < 0.05$ was considered significant.

RESULTS

Increased Uptake of Dietary Pi-Stimulated Lung Tumorigenesis in K-*ras*^{LA1} Mice

To determine the potential effects of high Pi on pulmonary tumorigenesis, tumor incidences in the lungs were measured. Our results clearly demonstrated that high dietary Pi signifi-

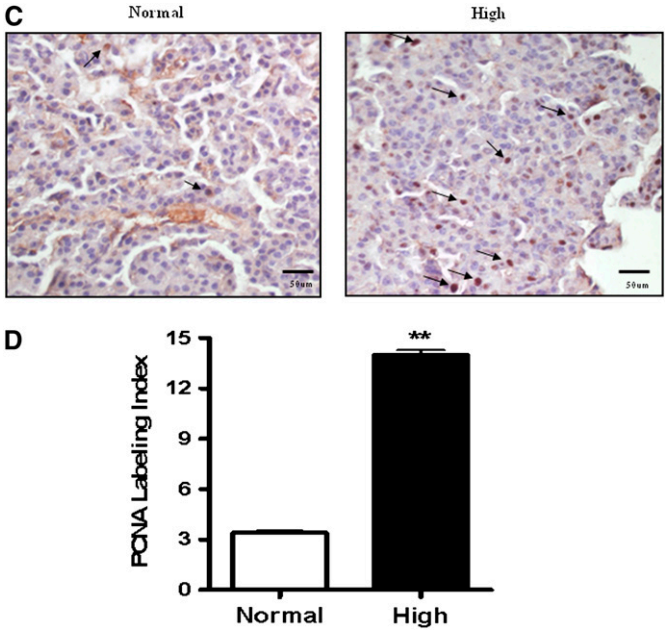

Figure 6. (*continued*)

cantly increased the lung surface tumor lesions as well as the size (at least 1.5 mm in diameter) (Figures 1A, 1B, and 1C). Histopathological examination also showed that pulmonary tumor progression was markedly stimulated by high Pi (*black arrows* and *dotted circles*, Figures 1D and 1E). Well-demarcated neoplastic nodules were clearly observed in the lungs of normal and high-Pi diet treated K-*ras*^{LA1} mice; however, the tumor mass of the high-Pi group was larger than that of the normal diet group (at least 1.5 mm in diameter). The above findings demonstrate that the adenoma number as well as tumor mass were significantly increased by high dietary Pi uptake.

Increased Uptake of Dietary Pi Elevated Serum Pi Levels and Pulmonary NPT-2b Protein Expression K-*ras*^{LA1} Mice

The diverse functional roles of Pi suggest that appropriate studies are needed to check the potential adverse effects of Pi on body weight gain. In our study, high dietary Pi did not cause significant effects on body weight gain (Figure 2A). However, high dietary Pi significantly increased the serum levels of Pi and PTH, whereas there was no statistically significant change in 1,25-$(OH)_2$ VD_3 serum levels. The serum Ca^{2+} level remained unchanged (Figures 2B and 2C). To confirm the hypothesis that phosphate transport in the lung may play a role in pulmonary tumorigenesis, the potential effect of high dietary Pi on lung NPT-2b protein expression was evaluated. As shown in Figure 3A, high Pi significantly increased pulmonary NPT-2b protein expression in K-*ras*^{LA1} mice. The increased NPT-2b protein expression was clearly demonstrated by densitometric analysis (Figure 3B).

Increased Uptake of Dietary Pi Stimulated Pulmonary Akt Activity

Because our previous studies demonstrated that high Pi altered Akt phosphorylation in the brain (32) and normal lungs in mice (8), we were interested in evaluating the potential effects of high dietary Pi on Akt and its related signals in terms of lung cancer progression. Our results indicated that high dietary Pi did not change the Akt protein expression and phosphorylation of Akt on Thr308 residue, however, high dietary Pi significantly increased phosphorylation of Akt on Ser473 residue (Figures 4A and 4B). Because Akt phosphorylation is associated with Akt kinase activity in lung cells (33), we performed an Akt kinase assay. As shown in Figures 4C and 4D, high Pi increased Akt kinase activity significantly in the lungs of K-*ras*^{LA1} mice. Moreover, high Pi significantly decreased the protein levels of the tumor suppressor, PTEN, and Akt binding partner, CTMP (Figure 4).

Increased Uptake of Dietary Pi Facilitated Cap-dependent Protein Translation in the Lungs of K-*ras*^{LA1} Mice

High dietary Pi increased Akt related cap-dependent protein translation in brain (32) and normal lung tissue of mice (8). Moreover, stimulated protein translation has been closely linked with cancer cell growth (33). Therefore, the potential effects of high Pi on protein translation on murine lung cancer were evaluated. For this reason, effects of high Pi on Akt downstream signal proteins such as p-mTOR, p-4E-BP1 were measured by immunoblotting. Activated Akt directly activates mTOR through phosphorylation of mTOR (34), and activated mTOR kinase is known to phosphorylate 4E-BP1 causing the dissociation of 4E-BP1 from eIF-4E. The free eIF-4E then binds to mRNAs, allowing cap-dependent translation (35). Our results showed that high Pi significantly increased the level of p-4E-BP1 protein in the lungs of K-*ras*^{LA1} mice (Figures 5A and 5B). In the next step, mTOR kinase assay and eIF4E immunoprecipitation assays were performed. High dietary Pi significantly increased mTOR activity (Figures 5C and 5D) and significantly decreased immune complex formation between eIF4E and eIF4E-BP1 (Figures 5E and 5F). Together, these results indicated that increased uptake of dietary Pi facilitated cap-dependent protein translation in the lungs of K-*ras*^{LA1} mice.

Increased Uptake of Dietary Pi Stimulates Cell Proliferation in the Lungs of K-*ras*^{LA1} Mice

Akt is known to regulate cell cycle progression (33) and taken with the above findings of Pi-induced Akt activity, prompted us to evaluate the effects of high Pi on cell cycle regulation in the lungs of K-*ras*^{LA1} mice. In addition, representative proteins important for cell cycle such as PCNA, cyclin D3, and cyclin-dependent kinase 2 (CDK2) were measured by Western blot analysis. Our results clearly demonstrated that high Pi significantly increased the protein expressions of PCNA, cyclin D3, and CKD2 (Figures 6A and 6B). IHC analysis of PCNA clearly showed that high Pi stimulated lung cancer cell proliferation in the lungs of K-*ras*^{LA1} mice (Figures 6C and 6D).

High Dietary Pi Significantly Increased Lung Tumorigenesis in Urethane-induced Lung Cancer Model Mice

The effects of high dietary Pi on lung tumorigenesis were confirmed in a second model of lung tumorigenesis, the urethane-induced lung cancer mouse. Results showed that high dietary Pi significantly increased the tumor development in urethane-induced lung cancer model mice (Figure 7). The mean number of tumor and the mean tumor diameter (at least 1.0 mm in diameter) were significantly increased by high dietary Pi (Figures 7A, 7B, and 7C). Histopathologic examination also demonstrated that pulmonary tumor progression was stimulated (Figures 7D and 7E).

DISCUSSION

Changes in serum phosphate might alter cell function by a number of mechanisms including transport into the cell. The mammalian NTP-2b transcripts were very recently found in relatively high abundance in total RNA prepared from lung

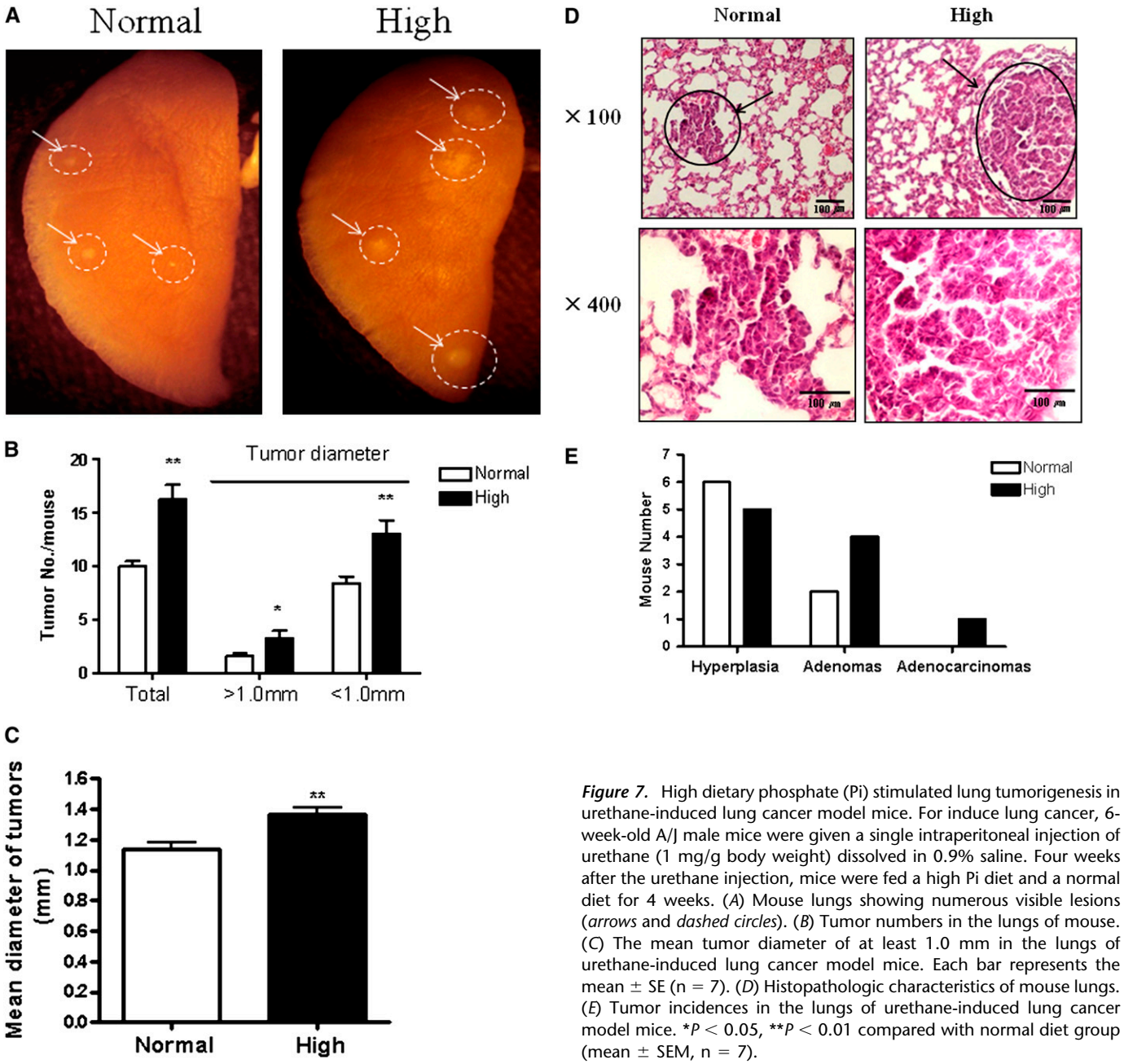

Figure 7. High dietary phosphate (Pi) stimulated lung tumorigenesis in urethane-induced lung cancer model mice. For induce lung cancer, 6-week-old A/J male mice were given a single intraperitoneal injection of urethane (1 mg/g body weight) dissolved in 0.9% saline. Four weeks after the urethane injection, mice were fed a high Pi diet and a normal diet for 4 weeks. (*A*) Mouse lungs showing numerous visible lesions (*arrows* and *dashed circles*). (*B*) Tumor numbers in the lungs of mouse. (*C*) The mean tumor diameter of at least 1.0 mm in the lungs of urethane-induced lung cancer model mice. Each bar represents the mean ± SE (n = 7). (*D*) Histopathologic characteristics of mouse lungs. (*E*) Tumor incidences in the lungs of urethane-induced lung cancer model mice. $*P < 0.05$, $**P < 0.01$ compared with normal diet group (mean ± SEM, n = 7).

tissues with restricted localization of expression to alveolar type II cells (36). Moreover, lung carcinomas derived from alveolar type II cells are found not only in bronchioloalveolar tumors but also in other types of adenocarcinoma and in large-cell carcinomas (37). Hashimoto and colleagues reported that NPT-2b may be a useful marker to analyze the histopathogenesis of lung cancer, and characterization of human adenocarcinoma by this probe is also expected (36). In addition, Yin and colleagues reported that NPT-2b is a potential target for immunotherapy of cancer with antibodies and vaccines (9). The fact that elevated Pi alters cell proliferation and gene expression and acts as a potentially important and novel signaling molecule, together with the poor prognosis of lung cancer involving lung-specific NPT-2b, prompted us to determine the effects of high dietary Pi on lung cancer progression in the lungs of K-*ras*^{LA1} mice.

In this study, therefore, the correlation between lung cancer growth and high dietary Pi in lung cancer-bearing K-*ras*^{LA1} mice was examined. Our study showed no body weight gain (Figure 2A). In fact, the AIN^{93} diet composition used in this study fulfills the requirement for normal growth in laboratory animals and thus excludes the possibility of malnutrition (26). However, high dietary phosphate increased NPT-2b protein expression (Figure 3) and serum phosphate levels significantly, whereas serum Ca^{2+} levels remained unchanged (Figure 2B). These data suggest that an increase of serum Pi may be associated with augmented NPT-2b protein expression without affecting Ca^{2+} homeostasis. In fact, our findings are supported by reports showing that serum Ca^{2+} levels are regulated within normal range strictly by increased endogenous PTH in response to changes in serum Pi levels from a high Pi diet (38). Interestingly, however, evidence suggests that PTH might be a cancer promoter (39). In fact, Yamamoto and colleagues report that PTH could activate Akt (40). Another report also demonstrated that activated Akt strongly stimulated cancer

progression (41, 42). Moreover, increased serum PTH levels were observed in lung cancer patients (43), and Niv and colleagues (44) reported that increased PTH levels were dependent upon the stage of the carcinoma. Taken together, these results suggest that increased PTH levels may be responsible for increased lung tumorigenesis found in our study.

Akt has become a favored second messenger from a therapeutic standpoint because numerous studies have demonstrated its role as a key molecule in malignant transformation. Evidence from several experimental models suggests that Akt is a key regulator of tumor development and progression (12, 45). In fact, Akt activation has been implicated in a variety of adverse cellular processes, including abnormal cell growth, inhibition of apoptosis, and abnormal gene regulation at both transcriptional and translational levels (46, 47). Recently, our *in vitro* study clearly demonstrated that high Pi stimulate the Akt pathway through control NPT expression in lung cells (15). Abnormal activation of Akt has been detected in human lung cancer precursor lesions and in established lung cancers as well (48, 49). Our results also clearly showed that high dietary Pi increased the phosphorylation of Akt at Ser473 while suppressing tumor suppressor PTEN as well as CTMP (Figures 4A and 4B). Enhanced Akt phosphorylation may be responsible for upregulation of Akt activity (Figures 4C and 4D) and thus stimulate lung tumor progression (Figure 1). Our results are supported by a recent report indicating that redundantly activated Akt could contribute to carcinogenesis in the lungs (50). Also, PTEN inactivation could accelerate K-*ras*-initiated lung tumorigenesis (51), and K-*ras* mutation-induced lung tumorigenesis could be suppressed by PTEN gene transfection (52). Moreover, CTMP acts as a negative regulator by suppressing Akt activity (53). Taken together, increased Akt activity and decreased protein expression of tumor suppressor PTEN and CTMP by high dietary Pi may promote lung tumor development observed in this study.

The mTOR protein is known to be involved in the Akt pathway and to control cell cycle progression (54), cell proliferation, and protein translation through its cell growth effectors p70S6K and 4E-BP1 (55, 35). The translation repressor, 4E-BP1, can block cap-dependent translation initiation by binding to eIF4E (56), and modification of protein translation is closely related with tumorigenesis (46). eIF4E is released from 4E-BP1/eIF4E complexes when 4E-BP1 is phosphorylated, and released eIF4E binds to the cap structure at the 5′ termini of RNAs, thereby allowing cap-dependent translation (35). Studies have demonstrated that eIF4E plays an important role in tumorigenesis. Overexpressed eIF4E induces malignant transformation by the activation of Ras (57) and promotes lung cancer formation (58) and progression (59). In addition, activated eIF4E is closely related to cancer chemotherapy resistance (60, 61). Moreover, Fingar and colleagues (57) also reported that cell size was significantly stimulated by overexpressed eIF4E through controlling cap-dependent translation. Several lines of research have demonstrated that activated Akt could induce activation of mTOR, and activated mTOR caused hyperphosphorylation of 4E-BP1 as well as translation of cyclin D (62, 63). Cyclin D is a cell-cycle regulatory subunit pairing with cyclin-dependent kinase (CDK) to principally govern cell-cycle progression (64). Our results revealed that high Pi increased mTOR kinase activity and 4E-BP1 phosphorylation (Figure 5). Moreover, high Pi also significantly increased protein expressions of cyclin D3, CDK2, and cell proliferation marker protein, PCNA (Figure 6). Together, these results suggest that high Pi promote lung cancer development through stimulating Akt-mediated cap-dependent protein translation and cancer cell proliferation.

Our study illustrated that high dietary Pi altered lung cancer development. Our results clearly demonstrated that increased uptake of dietary Pi stimulated pulmonary tumorigenesis parallel with Akt-mediated signals and suggest that careful regulation of dietary consumption of Pi may be critical for lung cancer prevention as well as treatment.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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