Chronic nasal exposure to nanoparticulate TiO₂ causes pulmonary tumorigenesis in male mice

Fashui Hong1,2,3 | Li Ji1,2,3 | Yingjun Zhou1,2,3 | Ling Wang4

1 Jiangsu Collaborative Innovation Center of Regional Modern Agriculture & Environmental Protection, Huaiyin Normal University, Huaian 223300, China
2 Jiangsu Key Laboratory for Food Safety and Nutritional Function, Huaiyin Normal University, Huaian 223300, China
3 Jiangsu Key Laboratory for Eco-Agricultural Biotechnology around Hongze Lake, Huaiyin Normal University, Huaian 223300, China
4 Library of Soochow University, Suzhou, China, Suzhou 215123, China

Correspondence
Fashui Hong, Jiangsu Collaborative Innovation Center of Regional Modern Agriculture & Environmental Protection, Huaiyin Normal University, Huaian, 223300, China.
Email: hfshui666@126.com

Funding information
The National Natural Science Foundation of China; Grant Numbers: 31671033, 81473007, 81273036 and 30901218); The National Natural Science Foundation of Jiangsu Province; Grant Numbers: BK20161306, and The Top-notch Academic Programs Project of Jiangsu Higher Education Institutions; Grant Numbers: PPZY2015A018.

Abstract
Chronic inhalation bioassays in rodents are used to assess pulmonary carcinogenicity for purposes of hazard identification and potentially for risk characterization. Numerous studies have been confirmed that exposure to titanium dioxide nanoparticles (TiO₂ NPs) may result in chronic pulmonary inflammation in both mice and rats. However, very few studies have focused on the pulmonary tumorigenesis. In this study, to examine whether chronic TiO₂ NP exposure induce tumorigenesis in the lung, forty mice (each group) were nasally exposed to 1.25, 2.5, and 5 mg/kg body weight TiO₂ NPs for nine consecutive months, lung pathology was then evaluated, and the biochemical function parameters in bronchoalveolar lavage (BAL) and tumor markers in the serum were investigated using an ELISA method. We observed that nasal exposure to TiO₂ NPs caused infiltration of inflammatory cells, tumorigenesis in the lung, and accompanied by significant increases of lactate dehydrogenase, alkaline phosphatase, and total protein levels in BLAF, significant increases in tumor markers including cytokeratin 19, neuron-specific enolase, carcinoembryonic antigen, squamous cell carcinoma antigen, and cancer antigen-125 in the serum. It implies that chronic inhaled TiO₂ NPs may increase possibility of pulmonary tumor formation for human. Therefore, the production and application of TiO₂ NPs should be paid more attention.

KEYWORDS
chronic inhaled exposure, mice, pulmonary tumorigenesis, titanium dioxide nanoparticles, tumor markers

1 | INTRODUCTION
Titanium dioxide nanoparticles (TiO₂ NPs), due to their larger surface to volume ratio, are widely used in industry for different applications, including food, clothing, electronics, cosmetics, medicine, agriculture and environmental decontamination of air, soil, and water, etc.1–4 However, the extensive use and development of TiO₂ NPs have intensified research efforts regarding toxicity.

Recently, TiO₂ NPs have been suggested to cause pulmonary toxicity of animals. For example, TiO₂ NP exposure resulted in increased levels of oxidative stress, pulmonary inflammation, and interstitial thickening of rats.5,6 Our previous studies also showed that TiO₂ NP exposure for 3 mo induced infiltration of inflammatory cells, pulmonary emphysema, edema, congestion, hemorrhage, thickening of the pulmonary interstitium, and apoptosis in mouse lung.7–9 Noël et al. suggested that TiO₂ NP exposure at 20 mg/m³ for 6 h induced lung inflammatory response, cytotoxic, and oxidative stress in male rats.10 Reference 11 showed that rats were exposed to bulk TiO₂ by inhalation exposure for 6 h/d, 5 d/wk for 2 y, suggesting rhinitis with squamous metaplasia in the anterior nasal cavity, cholesterol granulomas, bronchiolitis, bronchioloalveolar adenomas, and cystic keratinizing squamous cell carcinomas. Moreover, Lee et al. found that the lung tumors were different from common human lung cancers in terms of tumor type, anatomic location, tumorigenesis, and were devoid of tumor metastasis following exposure to bulk TiO₂.11 Although animal and human epidemiological data have led TiO₂ to be classified by the International Agency for Research on Cancer as “possibly carcinogenic to humans” by inhalation,12–14 however, few studies have examined the carcinogenicity of TiO₂ NPs.
NIOSH recently suggested that inhaled TiO2 NPs may be a potential occupational carcinogen, and they concluded that TiO2 NPs are not a direct-acting carcinogen, but acts through a secondary genotoxic mechanism. Heinrich et al. also found that rats exposed by inhalation to TiO2 NPs showed increased rates of adenocarcinomas, however, mice exposed to TiO2 NPs, according to the same methodology, did not exhibit differences in tumor rates compared to controls. Due to pressing concern regarding cancerogenic effects of TiO2 NP exposure on general and occupational populations and the limited number of in vivo studies on this topic, additional investigations are highly important.

In this study, mice were nasally exposed to 1.25, 2.5, or 5 mg/kg TiO2 NPs administered by nasal instillation for consecutive 9 mo. The aim of this study is to investigate whether chronic exposure to TiO2 NPs can result in tumorigenesis in the lung of mice and alterations of tumor-related parameters in the serum.

2 | MATERIALS AND METHODS

2.1 | Chemicals

The preparation, characteristics of TiO2 NPs including the anatase structure, size, surface area, mean hydrodynamic diameter, and ζ potential, have been described in our previously work. TiO2 NPs powder was dispersed onto the surface of 0.5% w/v hydroxypropylmethylcellulose (HPMC) that is a good dispersing agent and the suspension containing TiO2 NPs was treated ultrasonically for 30 min and mechanically vibrated for 5 min. The average particle size ranged from 5 to 6 nm, and the surface area was 174.8 m²/g. The mean hydrodynamic diameter of TiO2 NPs in HPMC solvent (5 mg/mL) ranged from 208 to 330 nm (mainly 294 nm), and the ζ potential after 24 h incubation was 9.28 mV, respectively.

2.2 | Animals and treatment

One hundred sixty 4-week-old CD-1 (ICR) male mice (22 ± 2 g body weight) were purchased from the Animal Center of Soochow University (China). All mice were housed in stainless steel cages in a ventilated animal room. Room temperature of the housing facility was maintained at 24 ± 2°C with a relative humidity of 60 ± 10% and a 12-h light/dark cycle. Distilled water and sterilized food were available for mice ad libitum. All animals were handled in accordance with the guidelines and protocols approved by the Care and Use of Animals Committee of Soochow University (China).

The male mice were randomly divided into four groups (N = 40 each), including a control group treated with 0.5% w/v HPMC and three experimental groups treated with 1.25, 2.5, or 5 mg/kg body weight TiO2 NPs, that is, 27.5, 55, 110 μg TiO2 NPs/mouse. The mice were weighed, volume of TiO2 NP suspensions was calculated for each mouse, and the fresh TiO2 NP suspensions were administered by nasal instillation every day for 9 mo. For dose selection, we consulted a report of the World Health Organization from 1969. According to the report, the LD 50 of TiO2 for rats is >12 g/kg body weight after oral administration. We also consulted that in November 2005, the United States National Institute for Occupational Safety and Health (NIOSH) proposed a recommended exposure limit (REL) for TiO2 NPs at 0.3 mg/m³. In Japan, the acceptable exposure concentration of TiO2 NPs was estimated to be 1.2 mg/m³ as a time weighted average (TWA) for an 8-h workday and a 40-h workweek. In Europe, food-grade TiO2 is approximately 36% of the TiO2 NPs that are smaller than 100 nm in at least one dimension, this exposure limit decreases to approximately 0.1 mg TiO2/person/d of nanoscale TiO2. Growth state, eating, drinking, and activity, or mortality were observed and recorded carefully daily during the 9 mo. After the 9-mo period, all mice were weighed, anesthetized with ether, blood samples were collected from the eye vein by rapidly removing the eyeball, and serum was collected by centrifuging the blood samples at 1200g for 10 min. The lungs were quickly removed and placed on ice and then dissected.

2.3 | Lung indices and tumor count

After weighing the body and lungs, the lung indices were calculated as the ratio of lung (wet weight, mg) to body weight (g). Lungs were perfused with cold phosphate buffer saline (PBS) and harvested, and tumors were scored using a dissecting microscope.

2.4 | Bronchoalveolar lavage (BAL) analysis

After blood collection, the lungs from the control and treated groups were immediately lavaged twice with phosphate buffer saline (PBS). An average of >90% of the total instilled PBS volume was retrieved both times and the amounts did not differ among the groups. The resulting fluid was centrifuged at 400g for 10 min at 4°C to separate the cells from the supernatant containing various surfactants and enzymes. The cell pellet was used for enumeration of total and differential cell counts as described by Reference 20. Macrophages, lymphocytes, neutrophils, and eosinophils recovered from the BALF were counted using dark field microscopy to assess the extent of phagocytosis. LDH, ALP, and total protein (TP) in the cell-free lavage fluid were analyzed using an automated clinical chemical analyzer (Type 7170A; Hitachi, Ltd., Tokyo, Japan).

2.5 | Titanium content analysis

The lung tissues (N = 5 each) were thawed and approximately 0.3-g samples were weighed, digested, and analyzed for titanium content using inductively coupled plasma-mass spectrometry (ICP-MS; Thermo Elemental X7; Thermo Electron Co., Waltham, MA).

2.6 | Histopathological examination of lung

Lung tissues from 30 mice each group were embedded in paraffin blocks, sliced to 5-μm thickness, and placed on separate glass slides (five slices from each lung). After hematoxylin–eosin staining, the sections were evaluated by a histopathologist unaware of the treatments, using an optical microscope (U-III Multi-point Sensor System; Nikon, Tokyo, Japan).
2.7 | Assay of tumor parameters

Serum levels of soluble fragment of cytokeratin 19 (CYFRA21-1) and neuron-specific enolase (NSE) were measured with immunoradiometric assay, serum levels of carcinoembryonic antigen (CEA), squamous cell carcinoma antigen (SCCag), and cancer antigen (CA)-125 were measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN). The absorbance was measured on a microplate reader at 450 nm (Varioskan Flash; Thermo Electron, Finland), and the concentrations of CEA, SCC, and CA-125 were calculated from a standard curve for each sample.

2.8 | Statistical analysis

The results were repeated five times. Data are presented as the means ±SD. One-way analysis of variance (ANOVA) followed by Tukey’s HSD post hoc test was performed to compare the differences of means among the multigroup data using SPSS 19 software (SPSS, Inc., Chicago, IL, USA). P value <0.05 was considered to indicate a statistically significant difference.

3 | RESULTS

3.1 | Body weight, lung indices, and titanium accumulation

Body weight and indices of mice are presented in Figure 1. As shown, an increased TiO2 NP dose led to a gradual decrease in body weight, whereas the lung indices were significantly increased (P <0.05). Furthermore, there was significant titanium accumulation with increased TiO2 NP dose (Figure 2, P < 0.05).

3.2 | Inflammatory cells and biochemical assessments in BALF

To further determine whether long-term TiO2 NPs exposure induces lung inflammation, we analyzed inflammatory cell content and biochemical changes in BALF. As shown, the numbers of macrophages, lymphocytes, neutrophils, and eosinophils (Figure 3), and LDH, ALP, and TP contents (Table 1) in the nano-TiO2-exposed mice showed obvious increases with increased TiO2 NP dose (P <0.05), indicating that TiO2 NP exposure caused severe inflammation and biochemical dysfunction in mouse lung.

3.3 | Histopathological lung evaluation

The histological changes in the lung specimens are shown in Figure 3. Unexposed lung samples exhibited intact architecture and arrange
regularity of pneumonocyte (Figure 4a), whereas those form TiO2 NP-treated mice exhibited severe pathological changes, including infiltration of inflammatory cells, tumorigenesis (Figure 4b–d). In addition, number of tumorigenesis, and tumor rates of mice were determined and show in Figure 5. With increased TiO2 NPs doses, these indexes were significantly elevated ($P < 0.05$), whereas the control did not observe tumorigenesis.

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>TiO2 NPs (mg/kg BW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>LDH (unit/L)</td>
<td>595.61 ± 31.53</td>
</tr>
<tr>
<td>ALP (unit/L)</td>
<td>110.49 ± 7.76</td>
</tr>
<tr>
<td>TP (g/L)</td>
<td>29.98 ± 2.73</td>
</tr>
</tbody>
</table>

* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Values represent means ± SD (n = 5).

### 3.4 Tumor parameters

Table 2 shows the effects of TiO2 NPs on levels of tumor-related parameters in mice. With increased TiO2 NP doses, there were significant increases in CYFRA21-1, NSE, CEA, CA125, and SCCag in the serum ($P < 0.05$). These were accompanied by tumorigenesis in mouse lung following chronic exposure to TiO2 NPs.

**FIGURE 4**  Histopathological observation of lungs of male mice after nasal instillation of TiO2 NPs for consecutive 9 mo (n = 5). Yellow cycle indicated inflammatory cell infiltration; blue cycle indicated tumorigenesis in the lung. [Color figure can be viewed at wileyonlinelibrary.com]
TABLE 2  Effect of TiO2 NPs on levels of tumor markers in male mouse lung by ELISA analysis after nasal instillation with TiO2 NPs for nine consecutive months

<table>
<thead>
<tr>
<th>Tumor parameter</th>
<th>TiO2 NPs (mg/kg BW)</th>
<th>Control</th>
<th>1.25</th>
<th>2.5</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYFRA21-1 (ng/g tissue)</td>
<td>95 ± 5.37</td>
<td>163 ± 4.81**</td>
<td>170 ± 9.85**</td>
<td>188 ± 11.29**</td>
<td></td>
</tr>
<tr>
<td>NSE (ng/g tissue)</td>
<td>215 ± 11.79</td>
<td>234 ± 12.86</td>
<td>293 ± 16.42*</td>
<td>319 ± 21.68*</td>
<td></td>
</tr>
<tr>
<td>CA125 (pg/g tissue)</td>
<td>191 ± 10.23</td>
<td>252 ± 13.61*</td>
<td>274 ± 11.46*</td>
<td>497 ± 27.58***</td>
<td></td>
</tr>
<tr>
<td>NSE (ng/g tissue)</td>
<td>247 ± 13.55</td>
<td>253 ± 10.27</td>
<td>389 ± 23.29**</td>
<td>408 ± 15.68**</td>
<td></td>
</tr>
<tr>
<td>CA125 (pg/g tissue)</td>
<td>191 ± 10.23</td>
<td>252 ± 9.61*</td>
<td>474 ± 21.46***</td>
<td>497 ± 17.58***</td>
<td></td>
</tr>
<tr>
<td>SCCag (ng/g tissue)</td>
<td>171 ± 9.59</td>
<td>225 ± 13.52*</td>
<td>239 ± 15.69*</td>
<td>262 ± 17.17*</td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05, ** P < 0.01, and *** P < 0.001. Values represent means ± SD (n = 5).

FIGURE 5  Numbers of tumorigenesis and tumor rats in 30 male mice after nasal administration with TiO2 NPs for nine consecutive months* P < 0.05. Values present means ± SD (n = 5). [Color figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

The photocatalytic properties of TiO2 NP exposure cause various toxic effects in the lungs in animals, however, whether chronic exposure to TiO2 NPs lead to pulmonary tumorigenesis is not reported.5–9,21,22 In this study, nasal administration of 1.25, 2.5, and 5 mg/kg of TiO2 NPs for consecutive 9 mo induced body weight reduction, increased lung indices, infiltration of inflammatory cells, and tumorigenesis in mouse lung tissues, and coupled with biochemical dysfunction that marked by increased LDH, ALP, and TP levels in the BALF, which also further supported the assertion that TiO2 NP nasal exposure induced pulmonary tumorigenesis. The discrepancies may be attributed to different types, exposure doses and times of TiO2 NPs etc. The pulmonary tumorigenesis caused by chronic exposure to TiO2 NPs may be involved in alterations of tumor markers in mice.

As we know, tumor markers are widely used in lung cancer management to aid diagnosis, to evaluate effectiveness of treatments, to monitor for recurrence after therapy and to predict prognostic information. As serologic markers for lung cancer management, CYFRA21-1, NSE, CEA, CA125, and SCCag are commonly measured. Molina et al.23 indicated that increased serum levels of CYFRA21-1, NSE, CEA, CA125, and SCCag were used in patients with lung cancer as an aid in histological diagnosis and prognosis. Reference 24 also showed that elevations of serum CEA, CYFRA21-1, and CA-125 levels are associated with worse prognosis in advanced lung cancer. To further confirm pulmonary tumorigenesis induced by chronic exposure to TiO2 NPs, in this study, we detected serum CYFRA21-1, NSE, CEA, CA125, and SCCag levels, suggesting significant increases in the tumor markers, which are consistent with pulmonary tumorigenesis of mice.

CYFRA21-1 is a cytokeratin expressed in simple epithelium such as the bronchial epithelium, and in malignant tumor derived from these cells.25 CYFRA 21-1 is demonstrated to be the most sensitive tumor marker for non-small-cell lung carcinoma (NSCLC), particularly squamous cell tumors.24,26–32 Therefore, increased CYFRA 21-1 concentration in the serum caused by TiO2 NPs was closely associated with pulmonary tumorigenesis of mice.

NSE is a glycolytic enzyme associated with neuroendocrine tumors. It is the tumor marker of first choice for small-cell carcinoma (SCLC),28,33 but increased serum NSE has been reported in patients with NSCLC.24–31 It implies that elevation of serum NSE level was due to pulmonary tumorigenesis in mice following exposure to TiO2 NPs.

In small quantities CEA is present in cells of normal tissues in healthy adults. Therefore, CEA is not usually present in the blood of healthy adults. CEA represents a heterogeneous group of glycoprotein whose specialized sialofucosylated glycol-forms serve as functional colon carcinoma L-selectin and E-selectin ligands, which may be critical to the metastatic dissemination of colon carcinoma cells.38–40 CEA is already demonstrated as a tumor marker in lung cancer.23,24,29,41,42 Although CA-125 is a mucin glycoprotein useful in the follow-up of ovarian cancer,43 increased CA-125 level has been suggested as a useful indicator of lung cancer.24,44–46 Therefore, our data suggested that increased serum CEA and CA-125 concentrations may be related to pulmonary tumorigenesis in mice following nasal exposure to TiO2 NPs.

Although SCC-ag is a tumor marker that appears to be very promising in the evaluation of cancers of the uterine cervix,47 it may be remarkably increased in squamous tumors of lung.48,49 Elevated serum
SCC-ag levels were also found in this study, which may be related to pulmonary tumorigenesis in mice caused by nasal exposure TiO2 NPs.

5 | CONCLUSION

This study suggests that nine months of nasal exposure of TiO2 NPs (1.25, 2.5, or 5 mg/kg) resulted in infiltration of inflammatory cells and pulmonary tumorigenesis of mice, coupled with high levels of serum CYFRA21-1, NSE, CEA, CA125, and SCCag. The pulmonary tumorigenesis may be associated with chronic pulmonary inflammation and impairment of biochemical function. Further studies related to the pulmonary tumorigenesis mechanisms of chronic nasal exposure to TiO2 NPs may needed to provide more evidences for its tumorigenesis. Our findings will be of benefit in understanding the effects of TiO2 NPs on the respiratory system and will focus attention on the effects of TiO2 NP application and exposure, especially long-term and low-dose nasal exposure on the human respiratory system.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (grant Nos. 31671033, 81473007, 81273036 and 30901218), the National Natural Science Foundation of Jiangsu Province (grant No. BK20161306), and the Top-notch Academic Programs Project of Jiangsu Higher Education Institutions (PPZY2015A018).

REFERENCES


How to cite this article: Hong F, Ji L, Zhou Y, and Wang L. Chronic nasal exposure to nanoparticulate TiO₂ causes pulmonary tumorigenesis in male mice. Environmental Toxicology. 2017;00:000–000. doi:10.1002/tox.22393.
学霸图书馆

www.xuebalib.com

本文献由“学霸图书馆-文献云下载”收集自网络，仅供学习交流使用。

学霸图书馆（www.xuebalib.com）是一个“整合众多图书馆数据库资源，提供一站式文献检索和下载服务”的24小时在线不限IP图书馆。

图书馆致力于便利、促进学习与科研，提供最强文献下载服务。

图书馆导航：

图书馆首页 文献云下载 图书馆入口 外文数据库大全 疑难文献辅助工具