RESEARCH COMMUNICATION

An Experimental Model for Induction of Lung Cancer in Rats by *Chlamydia Pneumoniae*

De-Jie Chu[1]*, Shui-Gen Guo[1], Chun-Feng Pan[1], Jing Wang[1], Yong Du[1], Xu-Feng Lu[1], Zhu-Yuan Yu[2]

Abstract

Objective: To assess induction effects of *Chlamydia pneumoniae* (Cpn) on lung cancer in rats. Methods: A lung cancer animal model was developed through repeated intratracheal injection of Cpn (TW-183) into the lungs of rats, with or without exposure to benzo(a)pyrene (Bp). Cpn antibodies (Cpn-IgA, -IgG, and -IgM) in serum were measured by microimmunofluorescence. Cpn-DNA or Cpn-Ag of rat lung cancer was detected through polymerase chain reaction or enzyme-linked immunosorbent assay. Results: The prevalence of Cpn infection was 72.9% (35/48) in the Cpn group and 76.7% (33/43) in the Cpn plus benzo(a)pyrene (Bp) group, with incidences of lung carcinomas in the two groups of 14.6% (7/48) and 44.2% (19/43), respectively (P-values 0.001 and <0.000 compared with normal controls). Conclusions: A rat model of lung carcinoma induced by Cpn infection was successfully established in the laboratory for future studies on the treatment, prevention, and mechanisms of the disease.

Keywords: *Chlamydia pneumonia* - benzo (a) pyrene - lung cancer - animal model - rat

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Introduction

Lung cancer is one of the most common malignant tumors threatening health and life, causing more deaths than any other malignant disease. The main etiology is smoking, air pollution, occupation factors, and human intrinsic factors such as family heredity. However, the exact nosogenesis remains unclear. Recent clinical epidemiological studies have suggested that an intimate relationship exists between Chlamydia pneumoniae (Cpn) infection and lung cancer incidence. Cpn is a common human respiratory tract pathogen. The perception on Cpn had been previously restricted to being the cause of community-acquired pneumonia. However, Cpn infection has been identified as a risk factor for lung cancer (Zhan et al., 2011). As a result, Cpn has become one of the hottest research points of the disease. Most scholars used clinical epidemiological research or serum gathering to determine the relationship between Cpn and lung cancer. Animal models of Cpn infections inducing lung cancer have not yet been reported. We verified our previous research on Cpn and lung cancer linkage using serum and tumor tissue from patients by designing a Cpn infection-induced lung tumor model in rats with or without benzo (a)pyrene (Bp), a carcinogen found in cigarettes. The results of the present study provide a new perception and method for lung cancer nosogenesis and therapy.

Materials and Methods

Cpn Strain

Cpn was provided by ATCC through Beijing Zhongyuan Limited. The strain TW-183 was preserved in a refrigerator at -70 °C before use. The Cpn strain concentration was adjusted to 6×10^6 IFU/mL (determined by experimental needs).

Animal treatments and methods

A total of 190 Wistar rats weighing 120 g to 130 g were purchased from the Shanghai Experimental Animal Center. This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Jinshan Hospital Affiliated to Fudan University. The animals were exposed to a seven-day circadian rhythm with free access to water and food, and then randomly divided into four groups. The control group had 40 rats, and the other three groups treated with Cpn, Bp, or Cpn+Bp had 50 rats each. The Cpn group was given a single injection of Cpn activity strains. The rats were anesthetized through intraperitoneal injection of 2% pentobarbital sodium (0.23 mL every 100 g body weight) and then were lain supine

[1]*Department of Pulmonary Medicine, Jinshan Hospital,* [2]*Zhongshan Hospital, Fudan University, Shanghai, China *For correspondence: dejiechu@yeah.net*

Table 1. The Incidence of Lung Cancer in Each Group

Group	Type of lung cancer (n)							
	n	Squamous	Adeno carcinoma	Large cell carcinoma	Small cell carcinoma	Total number of lung cancer	Incidence of lung cancer (%)	P value
1 (Cpn group)	48	2	4	0	1	7	14.6	0.001
2 (Bp group)	46	2	2	1	0	5	10.9	0.032
3 (Cpn+Bp group)	43	7	9	1	2	19	44.2	<0.001
Control group	40	0	0	0	0	0	0	

Table 2. Comparison of Infection Rates of Cpn in Three Groups

Group	n	Cpn-IgA(+)	Cpn-IgG (+)	Cpn-IgM(+)	Cpn (%)	P value
1 (Cpn group)	48	28	31	35	72.9	0.000
2 (Bp group)	46	0	0	0	0	
3 (Cpn+Bp group)	43	30	33	29	76.7	0.000

on the experiment table. The tongues of the rats were pulled gently to expose the larynx. Using an anatomical microscope to insert a plastic trocar under euthyphoria, we could determine whether the trocar had been correctly inserted into the endotrachea when the cotton fiber was blowing outside the trocar. An intratracheal injection of 100 μL Chlamydia pneumoniae TW-183 liquid bacteria (10^6 IFU/mL) was performed. Subsequently, the rat was immediately spun, and its chest was lightly pressed to allow even distribution of the liquid bacteria in the lung. The above procedure was repeated once a week for 2 months. The Bp group was given a single injection of Bp using an operating procedure adopted from a previously published method by Luo et al. (1995). The Cpn+Bp group was injected with Cpn and Bp. The control group was treated using the same procedure but using only an equivalent amount of saline.

Histopathological examinations

The rats were sacrificed, and a routine anatomical inspection was conducted to examine the tumor occurrences in the rats of each group. The gross morphology, color, hardness, inflammation, and necrosis of the lung tumor nodes were recorded. The tumor masses were fixed, sliced into sections, and then observed under a microscope to make precise pathological diagnoses.

Microimmunofluorescence

Serological diagnosis standards were as follows: a positive result was defined as titers of ≥ 1:32 for IgA and IgM and ≥ 1:64 for IgG (Fan et al., 2002).

Polymerase chain reaction (PCR)

Lung cancer tissues of rats positive for serum Cpn antibody were stored at low temperature. Approximately 1 g of tissue was made into a homogenate, and the DNA in the extractions was tested for Cpn using PCR. The procedure was performed according to the manufacturer's instructions. The oligonucleotide sequence of the primer was HL-1: 5'-GTT GTT CAT GAA GGC CTA CT-3' and HR-1: 5'-TGC ATA ACC TAC GGT GTG TT-3'. In the PCR procedure, the above primer would guide the synthesis and amplification of 437 bp segments. The following procedures were performed for amplification: degeneration for 5 min at 94 °C, followed by 35 PCR cycles of 94 °C for 1 min (DNA degeneration), 55 °C for 1 min (annealing), and 72 °C for 1 min (extension). PBS was used as the negative control, and Cpn TW-183 strains extracted from Cpn-DNA were used as the positive control. The amplification products were analyzed in 1.2% agarose gel electrophoresis according to the standard method. The strip position of the PCR amplification products was compared with that of a TaKaRa DL 2000 standard product to determine whether the product was coincident with the 437 bp DNA fragments (Chu et al., 2008).

Enzyme-linked immunosorbent assay (ELISA)

Tissues were partitioned into 6 μm slices after conventional treatment. An immunohistochemical assay was performed to detect specific Cpn antigens with specific Cpn-monoclonal antibodies.

Statistical analyses

Stata software was used to assess the numerical data with the x^2 test; $P < 0.05$ was considered to indicate statistical significance.

Results

Rat growth status

Except in the control group, the rats in the other three experimental groups died from anesthesia, contamination, or infection. After 3 months, 48, 45, and 43 rats from the Cpn, Bp, and Cpn+Bp groups survived, respectively. The body weights of the animals were monitored monthly. The changes in the body weights of the rats in the same cage exhibited no significant difference in the first 2 months. After 3 months, except for the negative control group, masses were observed in the lungs of a few rats. These masses were significantly larger than those in the other rats in the same cage ($P < 0.05$). Four months later, some of the rats died with a weight gain of up to 100 g. The surviving rats in each group were sacrificed at the end of the study on the 210th day.

Lung cancer incidence

No incidence of lung cancer was observed in the negative control group, whereas the other three groups showed different lung cancer incidences and sizes. The largest was 300 g, and the smallest was equivalent to the size of a rice grain. Table 1 shows the incidences of lung cancer in each group.

Pathology

Immediately after natural death, the rats were dissected,

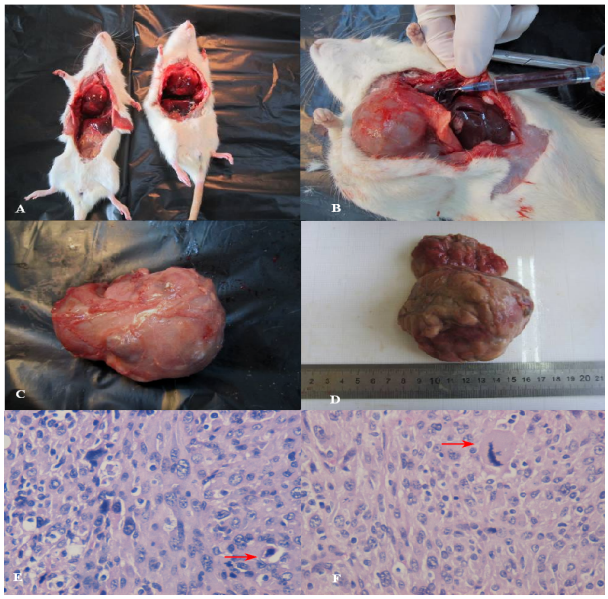

Figure 1. Results of Lung Cancer Induced with Cpn and the Pathologic Diagnosis. A and B: Profile of rats infected by Cpn. C and D: Resection specimen of lung carcinoma produced by Cpn. E: Tumor cell size differs, marked cytologic atypia, the arrow shows the giant nuclear Deformity tumor cells (HE staining x 400). F: The arrow shows typical pathological fission phase (HE staining x 400)

fixed, and sectioned then into slices for pathological diagnosis. The negative control group showed no cases of lung cancer. The mortality rates in the other three groups were detected through differences in lung cancer size. The largest tumor node had a diameter of 8 cm, whereas the smallest had a diameter of 0.1 cm. All tumors were gray and hard, with necrosis occurring at the middle of large nodules. The lung cancer incidences in the first, second, and third groups were 12, 5, and 19, respectively. Cpn-induced lung cancer and pathological diagnosis results are shown in Figure 1.

Serum Cpn antibodies

Cpn-IgM, -IgA, and -IgG were detected in rat serum from the first (Cpn or Bp) and third (Cpn or Bp) groups. Cpn-IgA and -IgG were significantly higher in the serum of Cpn-induced lung cancer rats than those in the negative control. The rats in the second group (Cpn or Bp) did not display serum Cpn antibodies. The results are shown in Table 2.

PCR

The PCR results of the lung cancer tissue of the first and third groups are shown in Figure 2.

Detection of Cpn antigen in lung cancer tissues

Lung cancer tissue from the second group (Bp) was found to lack the Cpn antigen, whereas the first (Cpn) and third (Cpn+Bp) groups were found to have Cpn antigens.

Discussion

Cpn, an obligate intracellular human pathogen, causes respiratory tract infections and constitutes a common cause of community-acquired pneumonia (Yen et al., 2005). It has been hypothesized to cause several chronic diseases, including atherosclerosis (Legan et al., 2004). Chronic Cpn infection is a risk factor of chronic obstructive pulmonary disease (COPD) (Zhou et al., 2011). Recent studies have shown that a high percentage of healthy blood donors harbor Chlamydia DNA and antigens (Karimi et al., 2010). Higher Cpn infection rates have been observed in the general population. In the study of Lin et al., the overall Cpn-IgG seropositive rate in 3,633 study participants was 45.5% (Lin et al., 2009). Such results have increased scientific attention on Cpn infections.

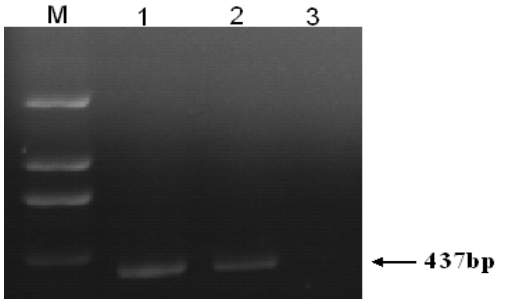

Figure 2. PCR detection of Lung Carcinoma Produced by Cpn infection. M: Marker (2000, 1000, 750, 500, 250, 100 bp); Lane 1: C. pneumoniae AR-39 as positive control; Lane 2: PCR product amplified from DNA of lung carcinoma produced by Cpn infection; Lane 3:negative control.

Many clinical epidemiological studies analyzing the association between Cpn infection and the risk of lung cancer have been reported (Jackson et al., 2000; Koyi et al., 2001; Anttila et al., 2003; Kocazeybek, 2003; Littman et al., 2004; Littman et al., 2005; Chaturvedi et al., 2010), but no clear consensus has been reached (Koh et al., 2005; Sessa et al., 2008; Smith et al., 2008). This relationship was assessed more closely by performing a meta-analysis (Zhan et al., 2011) based on publications collected from electronic databases such as Pubmed, Embase, Web of Science, and CNKI. Ultimately, 12 studies involving 2,595 lung cancer cases and 2,585 controls from four prospective studies and eight retrospective studies were included. Overall, people exposed to Cpn infection had an odds ratio (OR) of 1.48 [95% confidence interval (CI), 1.32–1.67] for lung cancer risk, relative to those not exposed. Cpn infection was clearly identified as a risk factor for lung cancer in all the prospective (OR, 1.16; 95% CI, 1.00–1.36) and retrospective studies (OR, 2.17; 95% CI, 1.79–2.63) reviewed, as well as in both the IgA ≥ 16 (OR, 1.22; 95% CI, 1.06–1.41) and IgA ≥ 64 cutoff groups (OR, 2.35; 95% CI, 1.88–2.93).

In conclusion, Cpn infection is associated with an increased risk for lung cancer, and a higher titer may be a better predictor of lung cancer risk. However, these studies used serum and not lung cancer tissue as the research material, and no Cpn infection animal model was used. A few articles about the relationship between Cpn and lung cancer have been published in China. A scholar from our department, Zhou et al. (2005), reported that the level of Cpn antigen in lung cancer tissues is significantly higher than that in normal lung tissues, suggesting that Cpn infection has some relevance to lung cancer. However, so far, no animal studies on Cpn-induced lung cancer have been reported. Therefore, animal experimental evidence

and molecular biological data are needed to support the speculated link between Cpn chronic infection and lung cancer. With assistance from the Shanghai Science and Technology Commission Fund, we investigated a rat model of Cpn-induced lung cancer. The rates of infection were 72.9% in the Cpn infection group and 76.7% in the Cpn+Bp infection group. Compared with the control group, the lung cancer incidences in the two infected groups were 14.6% and 44.2%, respectively, with P-values of 0.001 and 0.000. We detected Cpn DNA in rat lung cancer tissue, which were Cpn antibody and Cpn antigen-positive in the Cpn group and Cpn antigen-positive in the Cpn+Bp group. This result demonstrates that Cpn infection is closely linked with lung cancer in rats. The lung cancer incidence in the BP group was 10.9%, which is consistent with the results from Luo's study (Luo et al., 1995). The P-value was 0.032 compared with the control group. Bp is an important carcinogen in cigarettes. In the present study, the lung cancer incidence in the Cpn group was higher than that in the BP group. However, no significant differences were found between the Cpn and BP groups (P=0.075). Lung cancer incidence in the Cpn+BP group was significantly higher than that in the BP group (P=0.000). Thus, Cpn chronic infection is another independent risk factor for lung cancer. Smoking is a commonly recognized trigger factor for lung cancer incidence. The coexistence of smoking and Cpn chronic infection will thus have superimposed effects and lead to greatly increased lung cancer risk.

A recent study showed that Cpn infection rates in COPD can reach up to 60.9% (Chu et al., 2008), indicating a correlation. Boelens et al. (2011) reported that a relationship exists between squamous cell lung cancer and COPD. Punturieri et al (2009) reported that the presence of COPD increases the risk of lung cancer by up to 4.5-fold. However, the mechanisms by which Cpn chronic infection induces lung cancer remain unclear. Given that macrolide antibiotics have a remarkable therapeutic effect on Cpn infection, Cpn infection is controllable and even preventable. In conclusion, Cpn chronic infection is a new pathogenic factor leading to lung cancer. The establishment of a Cpn chronic infection-induced lung cancer in a rat model provides a powerful tool for the treatment and prevention of lung cancer.

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