

4-Nitroquinoline-1-oxide effects human lung adenocarcinoma A549 cells by regulating the expression of POLD4

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Abstract. The aim of the present study was to explore the expression of POLD4 in human lung adenocarcinoma A549 cells under 4-nitroquinoline-1-oxide (4NQO) stimulation to investigate the role of POLD4 in smoking-induced lung cancer. The lung cancer A549 cell line was treated with 4NQO, with or without MG132 (an inhibitor of proteasome activity), and subsequently the POLD4 level was determined by western blot analysis. Secondly, the cell sensitivity to 4NQO and Taxol was determined when the POLD4 expression level was downregulated by siRNA. The POLD4 protein levels in the A549 cells decreased following treatment with 4NQO; however, MG132 could reverse this phenotype. Downregulation of the POLD4 expression by siRNA enhanced A549 cell sensitivity to 4NQO, but not to Taxol. In conclusion, 4NQO affects human lung adenocarcinoma A549 cells by regulating the expression of POLD4.

Introduction

Lung cancer is the main type of cancer-related fatalities worldwide, and there is an extremely strong association between smoking and the formation of lung cancer (1,2). Tobacco is a chemical compound that contains >5,000 types of chemicals (3), of which 73 types were identified as carcinogens by the International Agency for Research on Cancer. There are >20 types that are associated with lung cancer, including polycyclic aromatic hydrocarbons, such as benzopyrene and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (4,5).

Studies have investigated the mechanism of smoking-induced lung cancer for decades, and its mechanisms include genetic alterations such as p53, KRAS mutations caused by forming adducts with DNA replication, activation of cell surface receptors, AKT, PKA-induced apoptotic genes, and the direct inhibition of the activity of tumor suppressor genes by tobacco, undermining the balance of the activity between oncogenes and tumor suppressor genes. However, the main mechanism is the formation of DNA adducts (6).

Benzopyrene is the most studied carcinogen in the tobacco-related carcinogen, which causes DNA damage by DNA adduct formation, and the specific DNA adducts have been detected in the lungs of smokers (7). DNA adducts can be cleared by DNA repair systems [such as nucleotide excision repair (NER)] (8), and intracellular DNA adducts can be rapidly cleared by effective intracellular DNA repair. While DNA is replicated, if the damaged DNA can not be repaired, DNA polymerase will terminate the copying, thereby preventing replication of damaged DNA or cell death. When there is more DNA polymerase than DNA adducts, this leads to gene mutations caused by mismatches. The clearance of DNA adducts mainly depend on NER, in which DNA polymerase has an irreplaceable role. DNA polymerase δ (Pol δ) is essential for DNA replication, which consists of four subunits, which are p125, p50, p68 and p12. The expression of the smallest DNA Pol δ subunit, POLD4, was low in certain non-small cell lung cancers and small cell lung cancers in our previous study, and the lack of POLD4 protein expression or downregulation of its expression by siRNA weakened DNA replication and the NER capability (9).

The p12 subunit is rapidly degraded in cultured human cells by DNA damage or replication stress induced by treatments with ultraviolet (UV) radiation, methyl methanesulfonate, hydroxyurea and aphidicolin (10,11). It is well known that there is a close association between smoking and lung cancer, and we hypothesize that smoking promotes the cancer risk by decreasing POLD4 expression.

4-Nitroquinoline 1-oxide (4NQO) is a quinoline derivative and a tumorigenic compound used in the assessment of the efficacy of diets, drugs and procedures in the prevention and treatment of cancer in animal models. It induces DNA lesions usually corrected by nucleotide excision repair. In the present study, 4NQO protein expression was observed in lung cancer

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cell line A549, which was treated by the major carcinogen in tobacco, benzopyrene analogs, in order to explore the mechanism of POLD4 in smoking-induced lung cancer.

Materials and methods

Cell culture. The human lung cancer A549 cells were obtained from the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum. All the cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C.

Transfection. Transfection was carried out using 50 nmol/l of a siRNA (POLD4 siRNA: Sense, 5-GCAUCUCUAUCCCCU AUGATT-3 and antisense, 5-UCAUAGGGGAUAGAGAUG CTT-3). Duplex (Sigma-Aldrich, St. Louis, MO, USA) targeting POLD4 mRNA or negative control 1 (control: Sense, 5-CUU UAAGCUCCCUGACGUUU-3 and antisense, 5-ACGCUC AGGGAGCUUAAAGUG-3, Ambion, Carlsbad, CA, USA) were assessed with Lipofectamine[®] 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA).

4NQO and MG132 treatments. The A549 cells were treated with different concentrations of 4NQO (0, 0.1 and 0.4 μmol/l) for 48 h, and were subsequently washed three times with phosphate-buffered saline prior to protein analysis.

The degradation of p12 is due to an accelerated rate of proteolysis that is inhibited by the proteasome inhibitors, MG132 and lactacystin. At 3 h before 4NQO treatment, the proteasome inhibitor MG132 (10 mmol/l) was added to block the activation of calpain, and the cells were harvested for protein analysis.

MTT. The siRNA-treated A549 cells or non-treated cells were separately cultured in 200 μl of culture medium in 96-well plates at a density of 8,000 cells/well. The following day, the medium was replaced with medium containing 4NQO or Taxol, followed by incubation for 48 h. Viable cells were measured in triplicate using TetraColor One (Seikagaku Co., Tokyo, Japan) with reference to the viability of mock-treated cells.

Western blot analysis. The total cell lysates of treated cells were separated by electrophoresis on 12.5% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was subsequently stained with Ponceau S and cut into several pieces according to the molecular weight of each designed protein. The pieces of membrane were blocked with 5% w/v non-fat dry milk in Tris-buffered saline and Tween-20 (TBST) buffer [20 mmol/l Tris-HCl (pH 7.4), 150 mmol/l NaCl and 0.05% Tween-20] for 1 h at room temperature. The blots were subsequently incubated with individual primary antibodies (POLD4, 1:1,000; cat. no. H00057804-M10; Novus Biologicals, LCC, Littleton, CO, USA; α-tubulin, 1:5,000; cat. no. sc-8035; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), corresponding to each designed protein for 1 h at room temperature. After three 15-min washes in TBST, the blots were incubated with alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit immunoglobulin G (Pierce, Rockford, IL, USA) for 1 h and washed with TBST three times

for 10 min. The Perfect Protein Western Blot kit (Novagen, Madison, WI, USA) was used for signal generation.

Statistical analysis. The statistical software SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) was used to calculate the significance according to Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

4NQO reduces POLD4 protein levels in the lung cancer A549 cell line. Certain studies have shown that UV radiation or hydroxyurea treatment converts Pol δ *in vivo* to the three-subunit form lacking p12. In the present experiment, after 48 h treatment of A549 cells by 4NQO, the POLD4 protein expression was evaluated by western blotting and the expression of POLD4 in 4NQO-treated A549 cells significantly decreased. There was a positive correlation between the decrease and the 4NQO concentration (Fig. 1).

Low expression of POLD4 induced by 4NQO may be regulated by ubiquitination. POLD4 is degraded in response to DNA damage through the ubiquitin-proteasome pathway (12). In order to clarify whether POLD4 degradation is caused by 4NQO through the ubiquitin-proteasome pathway, cells were treated prior to 4NQO treatment by protease activity inhibitor MG132, and it was observed that protease activity inhibitors can reverse the decreased POLD4 protein levels caused by 4NQO (Fig. 2), indicating that the low POLD4 expression due caused by 4NQO may be via the ubiquitin pathway.

Low expression of POLD4 weakens the DNA NER ability. Exposure to tobacco smoke and UV radiation can result in various types of DNA damage and subsequently lead to cancer formation. 4NQO is the UV radiation-mimetic chemical, which has been thought to cause bulky DNA adducts and chromosomal aberrations in exposed cells, and 4NQO-induced DNA damage can be repaired by a global repair mechanism (13). Therefore, 4NQO-induced mutagen sensitivity assays have been used to study susceptibility in 4NQO-treated cells in the present study. siRNA interference was used to regulate the expression levels of POLD4 in A549 to observe sensitivity of siRNA-treated cells and non-treated cells to 4NQO and Taxol. The low POLD4 expression cells had a more enhanced sensitivity to 4NQO toxicity compared to the siRNA non-treated cells, indicating that low POLD4 expression weakened DNA NER capacity. However, there was no difference in the sensitivity to Taxol between the siRNA-treated cells and non-treated cells, indicating that POLD4 expression is not involved in the sensitivity to Taxol (P>0.05; Fig. 3 and Table I).

Possible mechanisms of POLD4 in the effects of 4NQO on A549 cells. The formation of DNA damage caused by tobacco inhalation decreased POLD4 expression levels. Low POLD4 expression can cause genomic instability through different mechanisms; by contrast, the repair of DNA damage caused by 3-4 benzopyrene in tobacco depends on the NER capacity of Pol δ, while low POLD4 expression of the risk may weaken the NER capacity, thereby increasing the instability of the genome. These are two reasons for the cause of genomic

Table I. Taxol and 4NQO-induced mutagen sensitivity assays.

Treatments	Control	SiD4	F	P-value
Taxol, nmol/l				
0.5	70.0±2.0	75.0±2.6	6.9	0.059
2	35.7±2.1	40.3±2.5	6.1	0.069
8	6.3±1.5	6.3±0.6	0.0	1.000
4NQO, μmol/l				
0.1	95.7±2.5	85.0±2.6	25.6	0.007
0.4	79.3±2.1	53.0±3.6	145.1	0.000
1.6	39.7±2.5	10.0±1.7	282.9	0.000
6.4	9.3±1.2	4.3±0.6	45.0	0.003
12.8	3.0±1.0	3.0±0.8	0.0	1.000

Data are mean ± standard deviation. 4NQO, 4-nitroquinoline-1-oxide.

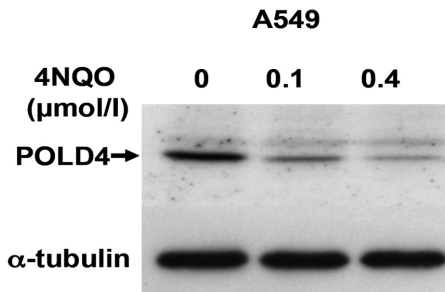


Figure 1. Decrease in POLD4 protein expression in lung cancer cell line A549 following 4-nitroquinoline-1-oxide (4NQO) treatment.

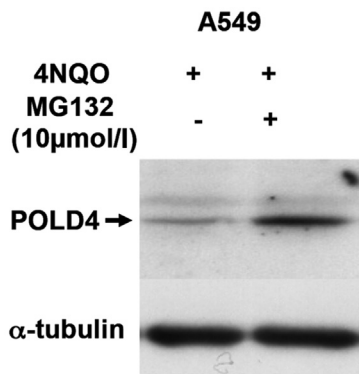


Figure 2. Protease activity inhibitors can reverse the decreased POLD4 protein levels caused by 4-nitroquinoline-1-oxide (4NQO).

instability and ultimately the increase in the risk (Fig. 4) of lung cancer formation.

Discussion

In previous studies, POLD4 expression in small cell lung cancer and a small section of non-small cell lung cancers is lower when compared to normal samples (9). Clinical data showed that almost all small cell lung cancers have a smoking history, indicating the possibility of a correlation between smoking-induced lung cancer and POLD4.

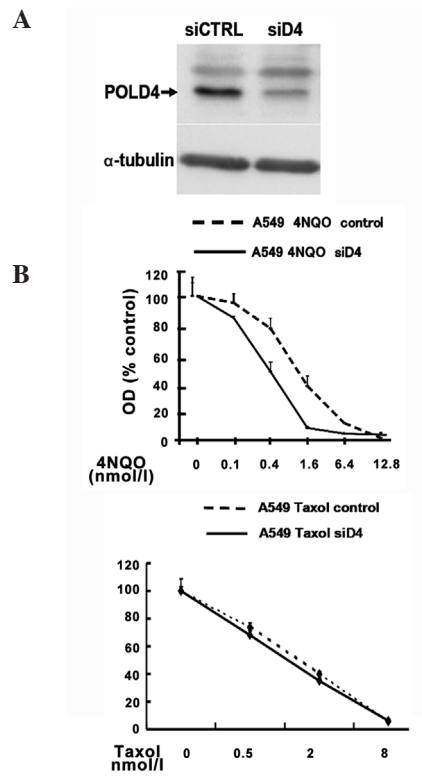


Figure 3. Low POLD4 expression weakens the nucleotide excision repair capability. (A) POLD4 protein levels decreased in the A549 cells by transfecting with POLD4 siRNA. (B) Low POLD4 expression enhanced cell sensitivity to 4-nitroquinoline 1-oxide (4NQO) toxicity, without affecting the sensitivity of cells to Taxol.

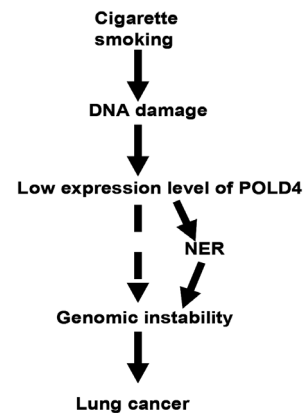


Figure 4. Possible mechanisms of POLD4 in the effects of 4-nitroquinoline-1-oxide on A549 cells. NER, nucleotide excision repair.

There are >20 types of lung cancer carcinogens in tobacco, of which the most significant carcinogens are polycyclic aromatic hydrocarbons such as benzopyrene and NNK. The most studied polycyclic aromatic hydrocarbon is benzopyrene. In the present study, the benzopyrene analogue 4NQO was used. 4NQO is a synthetic water-soluble carcinogen that is commonly used as a carcinogen in murine models for investigating the various stages of oral carcinogenesis. 4NQO exerts potent intracellular oxidative stress and its metabolic product binds to DNA predominantly at the guanine residues. These insults appear similar to the

damage induced by other carcinogens that are present in tobacco (14).

DNA Pol δ is involved in numerous DNA damage responses, and the Pol δ holoenzyme consists of four subunits, which are p125, p50, p68 and p12. The p12 subunit is known to be rapidly degraded in response to DNA damage by UV radiation, hydroxyurea or DNA replication stress, leading to the *in vivo* conversion of Pol δ 4 to Pol δ 3, a trimeric form lacking the p12 subunit (15), via the ubiquitin-proteasome pathway (10,11).

In our previous study (9), the low expression of POLD4 in small cell and non-small cell lung cancer suggested that there is a possible association between smoking and POLD4 genes. In the present study, 4NQO decreased the POLD4 protein expression in the lung cancer cell line A549, and the effect could be reversed by the proteasome inhibitor MG132.

4NQO produces a DNA adduct that is removed through Pol δ -dependent NER DNA. The effects of the decreased POLD4 expression were explored in the NER capacity in A549 cells, and the results showed that a low POLD4 expression enhanced the cell sensitivity to 4NQO, indirectly indicating that the POLD4 decrease weakened the NER capacity. In the present study, another type of cytotoxic drug, Taxol, was selected as a control to cause cytotoxicity, however, the repair of DNA damage did not depend on the NER capacity.

The study by Meng *et al* (11) showed that genotoxic agents, including UV and alkylating chemicals, induce a DNA damage response in which Pol δ 4 is converted to a trimer (Pol δ 3) by degradation of p12, and Pol δ 3 exhibits an enhanced ability for the detection of errors in primers and templates in comparison to its parent enzyme, whereas in the present study, a low expression of Pol δ 4 decreased the NER capacity.

In our previous study, we proved that regulation of DNA polymerase POLD4 influenced genomic instability in lung cancer, and found that downregulation of POLD4 in Calu6 cells results in G₁-S blockage through suppression of the AKT-Skp2-p27 pathway (9,16,17).

Our previous study also reported that the shRNA-mediated reduction of POLD4 resulted in a marked decrease in colony formation activity in Calu6, ACC-LC-319 and PC-10 cells, and POLD4 reduction was also associated with an increased population of karyomere-like cells, which may be an indication of DNA replication stress and/or DNA damage. siRNA-mediated reduction of POLD4 in cells with an abundant expression resulted in a cell cycle delay, checkpoint activation and an elevated frequency of chromosomal gap/break formation.

In conclusion, the present study reported that a significant tobacco carcinogen, benzopyrene analogue 4NQO, downregulated the POLD4 expression level, and thereby caused the decreased NER, further resulting in genomic instability, and ultimately an increased risk of lung cancer formation. Combined with the results of our previous study, we have proposed the mechanisms of action for POLD4 in smoking-induced lung cancer.

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