Thoracic Cancer

Open Access

Thoracic Cancer ISSN 1759-7706

ORIGINAL ARTICLE

Two polymorphic mutations in promoter region of DNA polymerase β in relatively higher percentage of thymic hyperplasia patients

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Keywords

DNA polymerase β; in vitro function study; mutation analysis; thymic hyperplasia.

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Received: 24 October 2020;
Accepted: 23 November 2020.

doi: 10.1111/1759-7714.13773

Thoracic Cancer **12** (2021) 588–592

Abstract

Background: DNA polymerase β is one of the key enzymes involved in the repair of DNA damage, and its high or low expression is closely related to tumorigenesis. In a previous study on lung cancer, we found three genetic mutations in the promoter region of the *Polb* gene could be detected in the Han Chinese population. The purpose of this study was to explore the relationship between these mutations and thymic hyperplasia.

Methods: Genomic DNA was extracted from 59 thymic hyperplasia patients by the salting out method and used for amplification of the promoter region of the *Polb* gene. The *Polb* gene mutation and its frequency were analyzed systematically by comparing them with the deposited wild-type gene sequence in the NCBI database. The three typical mutated sequences in the promoter region of *Polb* gene, -196G > T, -168C > A and -188_-187insCGCCC, were then amplified and ligated to pGL4.10 vector, so as to get the vectors used for the infection of 293T cells to explore their transcription activities by dual-luciferase reporter system.

Results: Two types of mutations, -168C>A and-188_-187insCGCCC, were found in a significantly higher percentage in patients with thymic hyperplasia than in normal healthy people after sequencing analysis of 59 patients and 60 healthy controls. These results suggest that the two mutations may be closely related to thymic hyperplasia. in vitro functional experiments showed that-168C>A could significantly increase promoter activity, whereas -188_-187insCGCCC could significantly reduce promoter activity, suggesting that these two mutations may affect the expression level of the *Polb* gene in cells.

Conclusions: Two types of mutations in the promoter region of the *Polb* gene, -168C>A and-188_-187insCGCCC, are associated with thymic hyperplasia and may become a new risk factor for this disease.

Key points

Significant findings of the study: Genetic mutations in the *Polb* gene are reported to be associated with different kinds of cancers. However, their relationship with thymic hyperplasia is still unclear.

What this study adds: For the first time, we report that two nucleotide mutations in the promoter region of the *Polb* gene are closely related with thymic hyperplasia after sequencing 59 patients and 60 healthy controls in the Han Chinese population.

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Introduction

DNA polymerase β is the smallest DNA polymerase in eukaryotic cells with a molecular weight of approximately 39 kDa. Its amino acid composition is highly conservative in the process of biological evolution and plays an important role in maintaining genetic stability.[1–3] The main function of DNA polymerase β is considered to be related to the process of DNA damage repair. It is involved in base excision repair (BER) in DNA repair and also participates in the 8-oxodG-induced mismatch repair process.[4,5] Abnormal expression and mutation of the *Polb* gene have been found in many human malignant tumors, such as colon, breast, gastric, bladder, prostate and esophageal cancers, suggesting that the abnormal expression of the *Polb* gene plays an important role in the occurrence and development of tumors.[6,7] Several studies have previously reported that the single nucleotide polymorphic mutations in the *Polb* gene is related to the occurrence and development of tumors.[8,9] However, our recent study indicated that few *Polb* gene mutations in lung tumor tissues could be detected in the Chinese Han population. On the contrary, we found that three mutations in the promoter region of the *Polb* gene could be detected in lung cancer patients, although there was no difference between the patient and common healthy subject groups.[10] In this study, we sequenced the promoter region of the *Polb* gene in 59 thymic hyperplasia patients and found that the frequency of two mutations, -168C > A and-188_-187insCGCCC, in patients was significantly higher than that in healthy individuals. Functional experiments in vitro also confirmed that these two mutations could significantly influence transcriptional promoter activity.

Methods

Study subjects

Hyperplastic tissues and adjacent degenerated normal thymus tissues from 59 patients, hospitalized in Beijing Hospital, were collected from June 2018 to December 2019. The samples were packed into 2 mL cryopreservation tubes within five minutes after dissection, quickly frozen in liquid nitrogen, and then stored at −80°C. All samples were confirmed by pathology after the operation, and all the subjects enrolled in the experiment had signed their informed consent. The median age of patients was 47.24 years old (15–73), including 31 males and 28 females. Control normal healthy subjects were enrolled in the Health Examination Center of Beijing Hospital with a median age of 42.53 years (25–69) and whole blood samples were collected for genomic DNA extraction and gene amplification. The detailed clinical information for thymic hyperplasia patients is shown in Table 1.

Genomic DNA extraction and gene amplification

Hyperplastic tissue was ground into powder under liquid nitrogen, and genomic DNA was extracted using the salting out method.[11] A 30 μL PCR reaction mixture was prepared as follows: 15μL 2 × Taq Plus Master Mix II (Vazyme Biotech, China), 0.2 μmol/L primer pairs (forward primer: 5′- GGAAACACAATCACCACAACCTT-3′; reverse primer: 5′-ACCAGCCTCGATTCTTGCTTT-3′) and 50 ng extracted genomic DNA. The amplification reaction was carried out as follows: predenaturation at 94°C for three minutes, followed by 30 cycles of denaturation at 94°C for 15 seconds, annealing at 63°C for 15 seconds, and extension at 72°C for one minute 45 seconds. Amplification was then separated by 2% agarose gel electrophoresis, and the target band (1.7kb) was recovered using a gel recovery kit (Biomed Biotechnology, China). Purified PCR products were sequenced using the sequencing primer (5′-TTCTCGGCATGGTTCACG-3′) by Beijing Tianyi Huiyuan Biotechnology Co., Ltd.

Analysis of sequencing results

The wild-type human *Polb* gene DNA sequence was downloaded from the NCBI database as the standard sequence (NC_000008.11) for sequence alignment. All chromatogram files were assembled and analyzed with the Seqman module of the Lasergene 7.2 program (DNASTAR, Inc., USA). Allele and genotype frequencies were calculated by the counting method, and a frequency comparison between patients and normal control healthy subjects was performed on the SPSS 12 platform using the X^2 test with the significance level set at 0.05.

Table 1 Clinical and pathological characteristics of enrolled patients

Gender	Number	%
Male	29	49.15
Female	30	50.85
Classification of MG		
EOMG	22	37.29
LOMG	37	62.71
OMG	16	27.12
GMG	43	72.88
AChR-Ab (+)	40	67.80
AChR-Ab (−)	19	32.20

AChR-Ab (−), AChR antibody-negative myasthenia gravis; AChR-Ab (+), myasthenia gravis with AChR antibodies; EOMG, early-onset myasthenia gravis; GMG, generalized myasthenia gravis; LOMG, late-onset myasthenia gravis; OMG, ocular myasthenia gravis.

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Plasmid construction

PCR products from the wild-type and mutated *Polb* gene carriers, -196G > T, -168C > A and -188_-187insCGCCC, were selected and used as the templates for the core promoter region (-1 to -314 upstream of the start codon, the first base of ATG is defined as +1) amplification with the following primer pairs: Forward: 5′-AAAA*CTCGAG*CTGGGCTGTCATTCTGAG-3′ (introduced XhoI restriction site is illustrated with underlined italics); Reverse: 5′-AAAC*AGATCT*GGCGGCCTGCACCCGAGA-3′ (BglII site is illustrated with underlined italics). After being digested with XhoI and BglII enzymes, the amplified fragment was ligated to the XhoI/BglII sites of pGL4.10 vector to yield the recombinant plasmid pGL4-polb.

Transcription activity determination using luciferase reporter system

293T cells (2×10^5 cells per well on 24-well plate) were resuspended and transfected with 600 ng pGL4-polb plasmid and 50 ng pRL-TK plasmid with liposome 2000 (Invitrogen, USA), following the manufacturer's recommended protocol. Six hours after transfection, cells were cultured with 500 μL fresh DMEM medium containing 10% FBS for an additional 48 hours. Cell lysates were prepared with passive lysis buffer and then used for the Firefly luciferase (Gluc signal) and Renilla luciferase (Rluc signal) activity measurement by the dual luciferase reporter assay system (Promega, USA). The transcriptional activity of each promoter was calculated and compared with the Gluc/Rluc ratio.

Results

Two types of mutations in promoter region of *Polb* gene associated with thymic hyperplasia

Similar to our previous report,[10] three types of mutations, -196G > T, -168C > A and -188_-187insCGCCC, were found in the promoter region of *Polb* gene after performing sequencing analysis of 59 patients with thymic hyperplasia and 60 healthy controls. Specially, the frequency of-168C >A and-188_-187insCGCCC in thymic patients was significantly higher than that in normal healthy people (Tables 2 and 3).

Table 2 Allele frequency of mutations found in the promoter region of the *Polb* gene in myasthenia gravis patients and normal healthy subjects

Group	*n*	-196G>T	-168C>A	-188_-187insCGCCC
Patients	118	10 (8.47%)	7 (5.93%)	4 (3.39%)
Normal	120	11 (9.17%)	3 (2.5%)*	2 (1.67%)*

*$P < 0.05$ indicates significant differences from the patient group after the student's *t*-test analysis.

Transcriptional activity of some mutated *Polb* promoters

Wild-type and three commonly detected mutated *Polb* promoters were subcloned into the promoterless vector pGL4.10 and used for transcriptional activity comparison in transfected 293T cells. It was found that the promoter activity of -196G>T exhibited little difference when compared with that of wild-type, whereas the promoter activity of -168C>A increased by about 30%, and -188_-187INSCGCCC mutant decreased by about 17%, relative to that of wild-type (Fig 1).

Discussion

The thymus provides a complex environment for the differentiation and maturity of T cells, and histological abnormalities in the thymus may result in the development of some serious autoimmune diseases, such as myasthenia gravis (MG).[12] MG is an autoimmune disease of the nervous system and it is believed to be mediated by the acetylcholine receptor antibody (AChR-Ab) which can cause the abnormal behavior of acetylcholine receptor (AChR) on the postsynaptic membrane at the neuromuscular junction.[13] In patients with early-onset MG (EOMG), 50%–60% show lymphoproliferative thymic hyperplasia, characterized by an ectopic germinal center (GC) and 15% of all the MG patients have thymoma, most of which are systemic MG.[14] It has been reported that thymoma may be involved in the occurrence of the disease through a variety of mechanisms, such as the expression of self-antigen in thymoma cells and the damage of negative selection of autoreactive T lymphocytes.[15] These data indicate that the abnormality of the thymus in patients with MG plays an important role in the production of AChR-Ab.

In MG, the initial inducement of thymic hyperplasia is still unclear at present,[16] but thymic hyperplasia is believed to be closely related to abnormal gene regulation. DNA polymerase β is the most critical polymerase in base excision repair (BER), which is an important repair system to maintain genomic integrity. Expression level of DNA polymerase β in normal mammalian cells is strictly controlled. Early genetic screening studies reported that a large number of mutated *Polb* variants could be detected in tumor tissues of lung,[17] gastric,[18] breast,[19] colorectal[20] and prostate cancers.[21] When *Polb* expression is too high or too low, it can lead to an increase in the rate of cell mutation, genetic instability and resistance to some anticancer

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Table 3 Genotype frequency of mutations found in the promoter region of the *Polb* gene in myasthenia gravis patients and normal healthy subjects

Group	*n*	-196G>T (heterozygous)	-196G>T (homozygous)	-168C>A (heterozygous)	-188_-187insCGCCC (heterozygous)
Patients	59	8 (13.56%)	1 (1.69%)	7 (11.86%)	4 (6.78%)
Normal	60	11 (18.33%)	NA	3 (5.00%)*	2 (3.33%)*

*$P < 0.05$ indicates significant differences from the patient group after the student's *t*-test analysis.

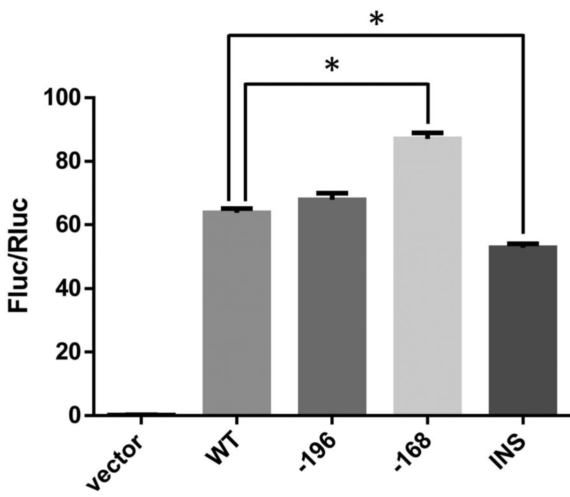

Figure 1 Transcriptional activity analysis of the *Polb* gene promoter. *$P < 0.05$ indicates significant differences from the wild-type. (■) vector; (■) WT; (■) −196; (■) −168; (■) INS

drugs.[17,22] Recently, we screened the promoter and all 14 exons of the *Polb* gene in 69 Chinese lung cancer patients, and only one nonsynonymous mutation, 587C>G, could be detected in the tumor tissues, although it was found in normal tissue and healthy control subjects. In addition to this type of mutation, three types of mutations (-196G > T, -168C > A and -188_-187insCGCCC) in the promoter region of the *Polb* gene were found in the Chinese Han population, although there was still no difference in their frequency between lung cancer and paracancerous tissues.[10] Similarly, three types of genetic mutation were also found in the promoter region of the *Polb* gene in this study (Table 2). Different to the data in lung cancer, it was found that two mutations, -168C > A and -188_-187insCGCCC in patients with thymic hyperplasia was significantly higher than that in normal controls, suggesting that these two mutations may be closely related to thymic hyperplasia (Tables 2 and 3). Further, in vitro functional tests showed that -168C > A could significantly increase promoter activity, while -188_-187insCGCCC could significantly decrease promoter activity (Fig 1). -196G > T and-168C > A were first reported in esophageal cancer tissue by Dong *et al.* from Zhengzhou University. in vitro experiments showed that both mutations could significantly enhance the activity of the *Polb* gene promoter and lead to cell resistance to the chemotherapeutic drug cisplatin.[23,24] In this study, it was found that only -168C > A could increase promoter activity, and promoter activity of -196G > T was similar to that of the wild-type. We believe that the difference in the cell lines used may be the main reason for the discrepancy between our data and that of the previous study. It has previously been reported that too high or too low expression of *Polb* gene will increase genomic instability, leading to carcinogenesis or drug resistance of cells.[17,22] Therefore, we speculate that the mutations in the promoter region of the *Polb* gene, -168C > A and-188_-187insCGCCC, can cause the up- or downregulated expression of the Polb protein, which induces the increase of genomic instability of thymocytes and directly or indirectly affects the onset or development of thymic hyperplasia.

In conclusion, in this study the relationship between thymic hyperplasia and *Polb* gene mutation was explored for the first time. It was found that two types of mutations in the promoter region of the *Polb* gene, -168C > A and-188_-187insCGCCC, were closely related to thymic hyperplasia and might be a new molecular risk factor for this disease.

Acknowledgments

This work was supported by grants funded by the Internal project of Beijing Hospital (BJ-2019-149), the National Natural Science Foundation of China (81971323) and the CAMS Innovation Fund for Medical Sciences (2018-I2M-1-002).

Disclosure

The authors had no conflicts of interest and no disclosures to declare.

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