# Relationship between COPD and polymorphisms of HOX-1 and mEPH in a Chinese population

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**Abstract.** Recent studies have proposed that susceptibility to chronic obstructive pulmonary disease (COPD) might be related with the polymorphisms of some genes encoding antioxidant enzymes, such as heme oxygenase-1 (HOX-1) and microsomal epoxide hydrolase (mEPH). We examined these polymorphisms in 256 patients with COPD and 266 healthy smokers from Han population in Southwest China. The frequencies of each allele were compared both individually and in combination between patients and controls. Polymorphisms of HOX-1 gene could be grouped into three classes: S (≤25 repeat), M (26-31 repeat), and L (≥32 repeat). The allele frequencies of class L and the genotypic frequencies of the group with L were significantly higher in COPD than in controls. Our findings also showed that the proportion of slow mEPH activity was significantly higher in COPD than in controls. Conversely, the proportion of fast mEPH activity was significantly lower in COPD. In combined analysis, the frequency of the individuals having at least one L allele in the HOX-1 gene promoter and slow or very slow activity genotype for mEPH was higher in COPD than in control. Genetic polymorphisms in HOX-1 and mEPH genes are associated with the development of COPD in Southwest China.

## Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by fixed and irreversible air-flow limitation, which is usually progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases. COPD is one of the leading causes of death worldwide, with an increasing prevalence and mortality. It is generally accepted that cigarette smoke is the most important risk factor for COPD. However, only 10-15% of smokers develop COPD (1). This phenomenon, together with the familial clustering of patients with early-onset COPD (2), strongly suggests that genetic factors may play an important role in the pathogenesis of COPD. One possibility is oxidant/antioxidant theory, which posits that oxidative stress initiates the onset of COPD whereas some antioxidant enzymes, such as heme oxygenase-1 (HOX-1) and microsomal epoxide hydrolase (mEPH) play a protective role in the lung (3).

Hox-1 is a key enzyme in heme catabolism and has been found to provide cellular protection against oxidant-mediated cellular injury (4). The $(GT)_n$ dinucleotide repeat in the 5'-flanking region shows length polymorphism, and has been demonstrated to modulate gene transcription under thermal stress (5) and associate with susceptibility to oxidant-induced apoptosis in lymphoblastoid cell lines (6). Therefore, it has been proposed and testified that this $(GT)_n$ repeat is associated with emphysema susceptibility induced by cigarette smoke in Japanese population (7). However, this issue was not confirmed by research in Canadian (8), Japanese (9) and American populations (10) and there has been no similar research in Chinese population hitherto.

mEPH is an enzyme essential for the metabolism of highly reactive epoxide intermediates produced by cigarette smoke (11). Two common polymorphisms have been detected in human mEPH gene: Tyr113→His at exon 3 and His139→Arg at exon 4, both of which could affect enzyme activity (12). It has been found that the slow metabolizing form of mEPH is significantly higher in the COPD group than in the controls (13-17). However, this issue still remains controversial (10), especially in East Asian populations (9,18-22). More data from these polymorphisms are necessary to test this association.

There is increasing evidence that the genetic susceptibility may depend on the coincidence of several gene polymorphisms acting together (23). Therefore, in the present study, we used a case-control design to analyze these polymorphisms of Hox-1 and mEPH gene both individually and in combination, and to test whether these polymorphisms influence

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Table I. Basic characteristics of the study groups.

| | COPD | Control | P-value |
|---|---|---|---|
| Subject | 256 | 266 | |
| Age (years) | 70.4±10.8[a] | 69.7±7.1 | 0.87 |
| Males/females | 210/45 | 208/58 | 0.96 |
| Smoking pack-years | 31.3±7.3 | 26.8±6.1 | 0.15 |
| Mean BI[b] | 448.4±127.2 | 441.0±116.9 | 0.89 |
| FEV1 (%) pred | 58.5±10.0 | 91.3±9.1 | <0.01 |
| FEV1/FVC[c] | 48.2±6.3 | 84.9±5.1 | <0.01 |

[a]Data are presented as mean ± SD. [b]Smoking history calculated by BI, Brinkman index (number of cigarettes/day x years). [c]Forced expiratory volume in one second/forced vital capacity.

the susceptibility to COPD in Han population from Southwest China.

## Patients and methods

*Study population*. Two hundred and fifty-six unselected smokers with COPD and 266 healthy smokers were involved in this study. COPD was defined by the results from multiple examinations including a medical history (symptoms of cough, sputum production or dyspnea, and/or a history of exposure to risk factors), a physical examination (hyperresonant chest and flattened hemidiaphragma), a chest roentgenogram (hyperinglation, flattened diaphragms, and marked loss of vascularity), a computed-tomography scan (areas of low attenuation) and pulmonary-function testing that demonstrated decreased FEV1:FVC ratios and impaired diffusion capacity (a post-bronchodilator FEV1 <80% of the predicted value in combination with an FEV1/FVC <70%) (24). COPD in the healthy smokers was excluded by Chest CT. Both patients and control were recruited from the First Affiliated Hospital of Kunming Medical College (Kunming, China) and belong to Han nationality from Southwest China. This study was approved by our institutional ethics committee, and informed consent for this study was obtained from all individuals. The detailed information of patients and controls is listed in Table I. The random selection criterion and the similar characteristics in age, gender ratio, smoking index indicated that population stratification was unlikely.

*DNA preparation*. Genomic DNA was extracted by phenol-chloroform method from whole peripheral blood leukocytes collected into EDTA (ethylenediaminetetra-aceticacide) tubes (25).

*Fragment analysis for HOX-1*. The Hox-1 gene 5'-flanking region containing poly $(GT)_n$ repeats was amplified by PCR with a fluorescently labeled dCTP (Applied Biosystems Inc., USA), a pair of primers (sense primer: 5'-ACGCCTGG GGTGCATCAAGTC-3'; anti-sense primer: 5'-GTGGGGTG GAGAGGAGCAGTCATA-3') 100 ng each other, 1 $\mu$l dNTP mixture (2.5 mM of each deoxynucleotide), and IU TaqDNA polymerase (Takara, Dalian, China). The PCR conditions executed in the thermal cycler (Eppendorf, Germany) were 30 cycles consisting of 94˚C for 1 min, 63˚C for 1 min, and 72˚C for 1 min. The sizes of the PCR products were determined by electrophoresis in ABI PRISM 377 sequencer (Applied Biosystems Inc., USA).

*Direct sequencing for mEPH exon 3*. A 514-bp fragment spanning position from 62 to 575 of the mEPH exon 3 was amplified by PCR with a sense primer (5'-GAAACTGCCTT GCCACTC-3') and an anti-sense primer (5'-CCTGCCTAGC TCTAAAGATG-3'). PCR was performed in 50 $\mu$l reaction mixture consisting of genomic DNA samples 250 ng, 5 $\mu$l 10X PCR buffer (20 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 2 $\mu$l dNTP mixture, 100 ng of each primer and 1 IU TaqDNA polymerase. Reaction conditions were as follows: 95˚C for 5 min, 35 cycles consisting of 94˚C for 1 min, 56˚C for 1 min, and 72˚C for 1 min, and finally 72˚C for 5 min. The PCR products were separated by electrophoresis in 1.5% agarose gel and then sequenced directly by using BigDye™ Terminator Cycle Sequencing kit and ABI PRISM 3700 sequencer (Applied Biosystems Inc.). The results were analyzed with DNAStar (DNAStar Inc., USA) software package.

*PCR-RFLP for mEPH exon 4*. A 210-bp fragment of exon 4 was amplified by PCR in 20 $\mu$l reaction mixture containing 100 ng of sense primer (5'-ACATCCACTTCATCCACGT-3') and anti-sense primer (5'-ATGCCTCTGAGAAGCCAT-3'), 1 $\mu$l dNTP mixture, and IU Taq DNA polymerase. Reaction conditions were as follows: 95˚C for 5 min, 30 cycles consisting of 94˚C for 30 sec, 57˚C for 30 sec, and 72˚C for 30 sec, and finally 72˚C for 5 min. The PCR products were incubated with *Rsa*I (Takara) at 37˚C overnight and then visualized on 3% agarose gel. The exon 4 mutated allele Arg139 produces an *Rsa*I restriction site and was cut to two fragments with 164 and 46 bp length, while the wild-type allele His139 remains uncut.

*Statistical analysis*. Using well-constructed power calculation software, Quanto (26,27), we were able to estimate that sample size in excess of 200 would be adequately powered to detect genetic association (assuming 90% power, $K_p$ 0.1, $R_G$ 2.6).

The uncalibrated Silverman test (28) was utilized to determine the mode of HOX-1 $(GT)_n$ repeat distribution and the null hypothesis was rejected when P<0.15.

To uncover any possible genetic risks for COPD, the frequencies of each allele and genotypes were compared both individually and in combination between patients and controls by two-tailed $\chi^2$ test. Odds ratios (OR) and 95% confidence intervals (CI) were also calculated to assess the relative disease risk conferred by a particular allele and genotype. Statistical analysis of age, sex, smoking history and pulmonary function test results was performed by unpaired t-test. Significance was accepted at P (probability) value <0.05. All the tests were performed using SPSS software (SPSS Inc., USA).

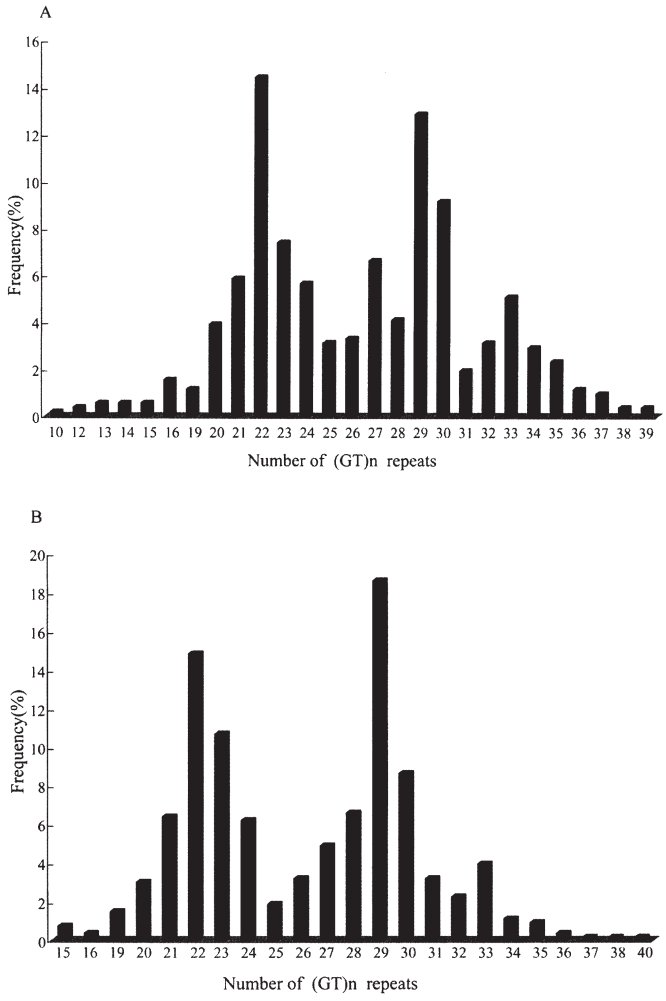


Figure 1. Frequency distribution of the number of $(GT)_n$ repeats in COPD (A) and control (B) groups.

## Results

Comparison of the observed genotypes of mEPH and those predicted by allele frequencies showed that the control group was in Hardy-Weinberg equilibrium (data not shown), indicating that control groups were sufficiently random and representative.

The number of $(GT)_n$ repeats in Hox-1 gene showed a distribution of 10-40 in the individuals studied (Fig. 1). The Silverman test rejected the unimodal distribution in COPD patients and the bimodal and four-modal distribution in controls (data not shown). To make the result uniform and comparable, a trimodal distribution was accepted, with three main peaks located at 22, 29, 33 repeats. Therefore, according to the number of $(GT)_n$ repeats, we divided the alleles into three subclasses: class S (≤25 GT repeats), class M (26-31 GT repeats), class L (≥32 GT repeats). An obviously higher allelic frequency of the class L was observed in patients with COPD than in the controls (16.4 vs. 9.2%, P=0.001, OR=1.9, 95% CI 1.3-2.8) (Table II). The M allelic frequency was slightly lower in the COPD group than in controls (38.1 vs 45.1%, P=0.024, OR=0.7, 95% CI 0.6-1.0), while the S allelic frequency was similar in both groups (45.5 vs 45.7%, P=1.0, OR=1.0, 95% CI 0.8-1.3). Furthermore, according to the Hox-1 genotypes, we divided the subjects into two groups: group I, individuals with at least one L allele (L/S, L/M, L/L), and group II, those without L allele (S/S, S/M, M/M). The genotypic frequencies of group I was significantly higher in COPD than in control group (28.1 vs. 16.9%, P=0.002, OR=1.9, 95% CI 1.3-2.9), while the genotypic frequencies of subjects in group II was similar in both groups (Table III).

The frequency of mEPH gene polymorphism was listed in Table IV. It is evident that the proportion of individuals heterozygous for His113 was significantly higher in the COPD group than in the controls (61.3 vs. 37.6%, P<0.001, OR=2.6, 95% CI 1.8-3.7). Conversely, the frequency of homozygous wild-type for Tyr113 was significantly lower in the COPD group than in the control group (21.1 vs. 44.4%, P<0.001, OR=0.3, 95% CI 0.2-0.5). However, there was no significant difference in genotype distribution of exon 4 polymorphism within these two groups. On the basis of the classification by Smith and Harrison (13), our subjects could be classified into four groups of putative mEPH phenotypes: normal, fast, slow and very slow (normal: homozygous wild-type for both exons 3 and 4, or heterozygous for both exons 3 and 4; fast: at least one mutation in exon 4 and no exon 3 mutation; slow: heterozygous for exon 3 and homozygous for exon 4; very slow: homozygous mutation type for exon 3; Table V). The present study showed that the frequency of the slow activity mEPH phenotype was significantly higher in patients with COPD than in controls (52.7 vs. 33.1%, P<0.001, OR=2.3, 95% CI 1.6-3.2). In contrast, the frequency of the fast activity mEPH phenotype was lower in the COPD group than in the control group (3.9 vs. 14.7%, P<0.001, OR=0.2, 95% CI 0.1-0.5).

Table II. Allele frequencies at the polymorphic locus for HOX-1 gene in the COPD and control groups.

| | No. of allele | | | Odds ratio | | |
|---|---|---|---|---|---|---|
| Allele class | COPD (%) | Control (%) | All other classes | S | M | L |
| L | 84 (16.4) | 49 (9.2) | 1.9 (1.3-2.8)[a] | 1.8 (1.2-2.7)[b] | 2.1 (1.4-3.1)[c] | 1.0 |
| M | 195 (38.1) | 240 (45.1) | 0.7 (0.6-1.0)[d] | 0.8 (0.7-1.1)[e] | 1.0 | |
| S | 233 (45.5) | 243 (45.7) | 1.0 (0.8-1.3)[f] | | | |

[a]P=0.001. [b]P=0.004. [c]P=0.000. [d]P=0.024. [e]P=0.232. [f]P=1.0.

Table III. Distribution of genotypes for HOX-1 gene in the COPD and control groups.

| Genotypes | HOX-1 genotypes L/L (%) | L/M (%) | L/S (%) | M/M (%) | M/S (%) | S/S (%) |
|---|---|---|---|---|---|---|
| COPD (n=256) | 12 (4.7) | 34 (13.3) | 26 (10.2) | 50 (19.5) | 61 (23.8) | 73 (28.5) |
| Control (n=266) | 4 (1.5) | 23 (8.6) | 18 (6.8) | 69 (25.9) | 79 (29.7) | 73 (27.5) |

| Group[a] | Group I (%) | Group II (%) |
|---|---|---|
| COPD (n=256) | 72 (28.1) | 184 (71.9) |
| Control (n=266) | 45 (16.9)[b] | 221 (83.1) |

[a]Comparison between subjects with S vs. that without S: 62.5 vs. 63.9%, P=0.78, OR=0.94, 95% CI 0.7-1.3; Comparison between subjects with M vs. that without M: 56.9 vs. 64.3%, P=0.09, OR=0.73, 95% CI 0.5-1.0; [b]28.1 vs. 16.9%, P=0.002, OR=1.9, 95% CI 1.3-2.9.

Table IV. Distribution of mEPH genotypes and phenotypes in normal and COPD groups.

| Genotypes | No. of individuals Homozygous wild-type (%) | Heterozygous (%) | Homozygous mutant (%) | P-value |
|---|---|---|---|---|
| Exon 3 polymorphism | | | | |
| COPD (n=256) | 54 (21.1) | 157 (61.3)[b] | 45 (17.6) | <0.001 |
| Control (n=266) | 118 (44.4)[a] | 100 (37.6) | 48 (18.0) | |
| Exon 4 polymorphism | | | | |
| COPD (n=256) | 204 (79.7) | 40 (15.6) | 12 (4.7) | 0.69 |
| Control (n=266) | 205 (77.1) | 49 (18.4) | 12 (4.5) | |

| Phenotype[c] | Fast (%) | Normal (%) | Slow (%) | Very slow (%) | P-value |
|---|---|---|---|---|---|
| COPD (n=256) | 10 (3.9) | 66 (25.8) | 135 (52.7)[e] | 45 (17.6) | <0.001 |
| Control (n=266) | 39 (14.7)[d] | 91 (34.2) | 88 (33.1) | 48 (18.0) | |

[a]Homozygous wild-type vs. others: P<0.001, OR=0.3-95% CI 0.2-0.5. [b]Heterozygous vs. others: P<0.001, OR=2.6-95% CI 1.8-3.7. [c]Phenotypes were determined by classification in Smith and Harrison (13). [d]Fast vs. others: P<0.001, OR=0.2-95% CI 0.1-0.5. [e]Slow vs. others: P<0.001, OR=2.3-95% CI 1.6-3.2.

When combined analysis was performed (Table VI), the combined genotypes of mEPH representing slow/very slow phenotype and Hox-1 group I were significantly higher in COPD group than in the control group (19.9 vs. 9.4%, P=0.001, OR=2.4, 95% CI 1.4-4.0). Conversely, the proportion of individuals with group II in the HOX-1 gene and fast or normal activity genotype for mEPH was lower in COPD patients (21.5 vs 41.4%, P<0.001, OR=0.4, 95% CI 0.3-0.6). There was no significant difference between the groups in combined comparison of slow/very slow phenotype in mEPH and HOX-1 group II or fast/normal phenotype in mEPH and HOX-1 group I (Table VI).

## Discussion

In the present study, we provided new genotyping data of Hox-1 and mEPH in Southwest Chinese population. Our study suggested a strong association between the $(GT)_n$ repeat number in the Hox-1 gene and susceptible to COPD in Southwest Chinese smokers in view of the higher frequency of the L allele as well as group I genotype in patients. Therefore, we speculated that the promoter activity in Hox-1 gene may be modulated by the length variability of the $(GT)_n$ repeats and people with L allele tend to show a defective or weaker detoxifying capability in the lungs by decreasing the promoter activity in Hox-1 enzyme. As a result, the smokers carrying the L allele may have a higher risk for COPD, while the smokers with the class M may have a lower risk for COPD. Our study confirmed the results of Yamada *et al* (7), but contradicted with other studies in Canadian population (8), Japanese (9) and American population (10). This discrepancy might result either from different selection of study subjects or a racial difference. In the Canadian and Japanese reports (8,9), all the subjects had decline of lung function or emphysema rather than COPD. An earlier onset of decline of lung function does not indicate symptoms of COPD or

Table V. mEPH phenotypes classification based on genotypes.[a]

| Phenotypes | Exon 3 | | | Exon 4 | | |
|---|---|---|---|---|---|---|
| | Tyr113/Tyr113 | Tyr113/His113 | His113/His113 | His139/His139 | His139/Arg139 | Arg139/Arg139 |
| Normal | + | | | + | | |
| | | + | | | + | |
| Fast | + | | | | + | |
| | + | | | | | + |
| Slow | | + | | + | | |
| | | + | | | | + |
| Very slow | | | + | + | | |
| | | | + | | + | |
| | | | + | | | + |

[a]Phenotypes were determined by classification in Smith and Harrison (13).

Table VI. Frequencies of combined genotypes for mEPH and HOX-1 gene in COPD and control groups.[a]

| Group | mEPH slow/very slow and HOX-1 group I (%) | Others (%) | OR (95% CI) | P-value |
|---|---|---|---|---|
| COPD (n=256) | 51 (19.9) | 205 (80.1) | 2.4 (1.4-4.0) | 0.001 |
| Control (n=266) | 25 (9.4) | 241 (90.6) | | |
| | mEPH fast/normal and HOX-1 group II (%) | Others (%) | OR (95% CI) | P-value |
| COPD (n=256) | 55 (21.5) | 201 (78.5) | 0.4 (0.3-0.6) | <0.001 |
| Control (n=266) | 110 (41.4) | 156 (58.6) | | |

[a]mEPH slow/very slow and HOX-1 group II vs. others: 50.4 vs. 41.7%, P=0.053, OR=0.7, 95% CI 0.5-1.0; mEPH fast/normal and HOX-1 group I vs. others: 8.2 vs. 7.5%, P=0.87, OR=1.1, 95% CI 0.6-2.1.

emphysema, and decline of lung function can be caused by other lung diseases besides COPD and emphysema, such as idiopathic pulmonary fibrosis, silicosis, lung tumor and even some diseases excluding primary lung tissue diseases. As far as racial differences are concerned, although both Chinese and Japanese are Mongolian, the frequency of group I (and L allele as well) in our control group was slightly lower than in the Japanese (16.9 vs. 20%) (7). The reason for this phenomenon might be that gene frequency varies among different racial groups. The discrepancy might also be due to exposure to different environmental factors, besides cigarette smoking, which may be important in the development of COPD.

Our results also indicated that mEPH might contribute to susceptibility to COPD in that people with the slow phenotype in mEPH gene may be more susceptible to COPD while those with the fast phenotype in mEPH gene may be of stronger ability to protect their lungs from oxygenic injury caused by cigarette smoking. This result confirmed the original research in Caucasian (13,16,17) and some in Mongolians (14,21) by and large, but contradicts with most East Asian ones (18-20). Besides the genetic background and different criterion on patient selection, this discrepancy might be due to the incorrect typing result and/or phenotype allocate. It has been reported that the PCR-RFLP technique, which was widely performed in most research on exon 3 (9,13,15-17,19-21), was not appropriate since the results were not consistent with those by direct sequencing (18). The biased frequencies obtained from this method might induce some wrong conclusion. Moreover, when the Smith and Harrison (13) criterion was utilized for phenotype classification (16,18-22), a formula could be deduced: the number of normal and fast phenotype - the number of homozygous wild-type genotype in exon 3 = the number of heterozygous genotype in exon 3 - the number of slow phenotype, both sides of which give a value indicating the number of genotype heterozygous for exons 3 and 4 (Table V). However, this formula could not be fulfilled in many data sets, such as control and emphysema groups in Smith and Harrison (13), emphysema group in Takeyabu *et al* (19), COPD groups in Rodriguez *et al* (16) and Zhang *et al* (20), which implied that multiple criteria were in use in previous research or that many phenotype frequencies might not be accurate. Lack of original data, unfortunately, hinder us from re-analyzing and obtaining an updated conclusion.

As a complex polygenic disease, it seems that the mechanisms of COPD are influenced by multiple gene actions and the genetic susceptibility may depend on the coincidence of several gene polymorphisms acting together. Therefore, we analyzed the genotypes of Hox-1 and mEPH genes in combination to find the association between these combined genotypes and COPD. Our study supported that genotype of slow/very slow activity in mEPH and Hox-1 group I was significantly higher in COPD group than in the controls and confirmed the original research in Japan in part (9). Conversely, the genotype of fast/normal activity in mEPH and HOX-1 group II was obviously lower in COPD patients. Thus, we speculated that persons with simultaneous mEPH slow/very slow phenotype and Hox-1 group I might have a higher risk of developing COPD, while the individuals with mEPH fast/normal phenotype and HOX-1 group II may have stronger ability to protect their lungs from oxygenic injury caused by cigarette smoking.

In conclusion, genetic polymorphisms of Hox-1 and mEPH genes, both individually and in combination, are associated with the development of COPD in a Southwest Chinese population. Further studies with large size in various ethnic populations as well as family studies are indispensable to clarify the underlying molecular and pathophysiological mechanisms in the development of COPD.

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