Impact of Alcohol Dehydrogenase Gene 4 Polymorphisms on Esophageal Squamous Cell Carcinoma Risk in a Chinese Population

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Abstract

Background

Esophageal squamous cell carcinoma (ESCC) is very common in China and is also one of the most common cancers worldwide. The purpose of this study was to examine the associations between genetic variants of various cancer-related genes and the risk of ESCC.

Methods

In this study, we first examined the association between 18 potentially disruptive genetic variants of 17 genes, including alcohol dehydrogenase 4 (ADH4) and checkpoint kinase 2 (CHEK2), and ESCC risk in a Hangzhou population of 617 patients matched with 534 controls. Among the 18 single nucleotide polymorphisms (SNPs), two were validated in a Jinan case-control set. Individuals with the ADH4 rs3805322 AA or AG genotype had ORs of 1.10 (95% CI = 0.81–1.49, P < 0.001) or 1.86 (95% CI = 1.33–2.59, P = 0.559),
respectively, for developing ESCC compared with individuals with the GG genotype. CHEK2 rs4822983 CC carriers showed a marginally significantly decreased ESCC risk compared with those carrying the CT and TT genotypes in the validation set (95% CI = 0.61–1.01, \(P = 0.064\)). However, no evidence of interaction existed between the two SNPs and smoking or drinking in the Jinan case-control set.

**Conclusions**

In conclusion, this current study provides substantial evidence that genetic polymorphisms of rs3805322 in the ADH4 gene may be associated with an increased risk of developing ESCC in two Chinese Han populations. Future studies to address the biological function of this polymorphism in the development of ESCC are warranted.

**Introduction**

Esophageal cancer (EC) is regarded as one of the most common and fatal malignant tumors in the world. More than 90% of esophageal cancers are esophageal squamous cell carcinomas (ESCCs), which is the most common pathologic type in developing nations [1]. ESCC has a relatively high incidence and morbidity in China compared with western countries [2–4]. Accumulating epidemiological evidence indicates that tobacco smoking, substantial alcohol intake, micronutrient deficiency, and dietary carcinogen exposure can greatly increase the risk of developing squamous cell carcinoma [5]. All these factors can induce or enhance DNA damage, which initiates and/or promotes carcinogenesis. DNA repair has been recognized as the most critical mechanism of protection against DNA damage. Various genes are involved in alcohol-associated carcinogenesis and DNA repair. Single nucleotide polymorphisms (SNPs) in genes such as checkpoint kinase 2 (CHEK2) [6] and nei endonuclease VIII-like 2 (NEIL2) [7], which contribute to inter-individual diversity in DNA repair capacity, may play a significant role in modifying EC risk [8–10].

The occurrence and development of EC is a multi-stage and multi-factor process involving the accumulation and interaction of various environmental factors and genes. Research on EC-related genes has established that alcohol dehydrogenase 4 (ADH4) [11, 12], fibroblast growth factor receptor (FGFR) [13], thymidylate synthetase (TYMS) [14], and cyclin-dependent kinase inhibitor 1A (CDKN1A) [15, 16] are directly involved in EC to various degrees. Genetic variants of mucin 1 (MUC1) [17] and S100 calcium binding protein A14 (S100A14) [18] have been reported to be associated with ESCC. In addition, abnormal expression of ABI family, member 3 binding protein (ABI3BP) [19]; klotho beta (KLB) [20]; long non-coding RNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) [19]; and particular microRNAs, including miR-1206 [20] and miR-612 [21], has been observed in cancer cells.

Considering the importance of these genes in ESCC, we conducted a large case-control study to estimate the association between 18 potential functional genetic variants of 17 genes and ESCC risk in a discovery dataset (Hangzhou population) and further validated two SNPs in a validation dataset (Jinan population).

**Materials and Methods**

**Study subjects**

To estimate the frequencies of the alleles and genotypes of newly identified polymorphisms, 617 southern Han Chinese patients with ESCC and 534 sex- and age-matched (±5 years)
control subjects were recruited from Zhejiang Cancer Hospital (Hangzhou, Zhejiang Province, China) between January 2012 and November 2013. Control subjects were individuals who were seeking health care in the same hospital for non-oncologic diseases. For the validation arm, 540 northern Han Chinese patients with ESCC were recruited between June 2009 and April 2012 from Shandong Cancer Hospital (Jinan, Shandong Province, China). These patients presented with histologically confirmed ESCC. Five hundred and fifty cancer-free control subjects, randomly selected from a cancer-screening program for the early detection of cancer performed in Jinan city, were frequency-matched to the cancer cases by age (±5 year), gender, and residential area. A short questionnaire was used to obtain demographic and risk factor information, including smoking and alcohol status. Smokers were classified as individuals who smoked once per day for more than one year. Subjects were also defined as alcohol consumers if they ingested alcohol at least once per week. Written informed consent was obtained from all the patients enrolled in this study. The study was approved by the ethics review board of Zhejiang Province Cancer Hospital.

SNP selection and genotyping

We selected SNPs based on their functional potential with a minor allele frequency greater than 0.05 in the Asian population and reviewed related literature to identify potential SNPs that could impact EC.

After signing informed consent forms, each subject donated 5 ml of peripheral blood, which was used for genomic DNA extraction. A Blood Genomic DNA Isolation Kit (Axygen Scientific Inc., CA, USA) was used to extract DNA from leukocyte cell pellets according to the manufacturer’s instructions. The DNA purity and concentration were determined by spectrophotometry. All 18 SNPs that were detected at the first stage in the Hangzhou case-control set (discovery set) were also detected using the MassARRAY system (Sequenom Inc., San Diego, California, USA). The genotyping of rs3805322 (ADH4) and rs4822983 (CHEK2) in the Jinan case-control set was performed using TaqMan assays on an ABI 7900 system (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Primers and probes for the two SNPs were supplied by Applied Biosystems. Real-time quantitative polymerase chain reaction (qPCR) was performed under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, and 45 cycles of 95°C for 30 seconds and 60°C for 1 minute. A random selection of fifteen percent of the samples was reciprocally tested by different persons, and the reproducibility was 99.5%.

Statistical Analysis

Differences in gender, age, lifestyle habits and genotype distributions between patients and control subjects were evaluated using Pearson’s Chi-Square (X²) test. Multiple correction (Bonferroni correction) was used to validate the significant variables. The dominant and recessive models were used to assess the risk of SNP genotypes in EC. The reference group was the minor homozygous genotype among the controls. The associations between two SNPs and the risk of EC were estimated by computing the odds ratios (ORs) and their 95% confidence intervals (CIs) from both univariate and multivariate logistic regression analyses. The ORs were also adjusted for age, gender and smoking status where appropriate. The gene-environment interaction was evaluated by one-way analysis of variance. All the tests were two-sided, and P < 0.05 was considered statistically significant. All the statistical analyses were performed with the SPSS software package (SPSS 18.0 Inc., Chicago, IL, USA).

Results

The clinical characteristics of the discovery dataset (Hangzhou case-control set) and the validation dataset (Jinan case-control set) are listed in Table 1. These two case-control sets were used
to detect associations between five ADH1B-ADH1C-ADH7 cluster SNPs and the risk of ESCC [8]. There was no difference in the sex or age distribution between ESCC patients and healthy controls for both the Hangzhou and Jinan populations. There were more smokers and alcohol drinkers among the ESCC cases in the Jinan population compared with the controls (both P < 0.05). Unfortunately, no data were collected on the smoking and drinking habits of the controls in the Hangzhou population. The Hardy-Weinberg equilibrium (HWE) values of these 18 SNPs in the Zhejiang set are presented in Table 2. In addition, the P values of the HWE tests for rs3805322 (ADH4) and rs4822983 (CHEK2) in the controls of the Jinan set were 0.495 and 0.873, respectively.

We genotyped 18 selected SNPs in 17 genes for all 617 ESCC patients and 534 control subjects in the discovery arm (Hangzhou). The location and potential effects of these 18 SNPs, some of which cause amino acid substitutions in the proteins, are listed in Table 2. The genotype frequencies of rs3805322 (ADH4) polymorphisms were significantly different between cases and controls (P < 0.001, Table 2). Sixteen SNPs in 15 genes, including CHEK2, MBL2, MALAT1, and ABI3BP, showed no significantly different allele frequency distributions between ESCC patients and control subjects based on Fisher’s exact test (Table 2). Unconditional univariate logistic regression analysis revealed a significantly increased risk of developing ESCC in subjects with the AA genotype for rs3805322 (ADH4) compared with those with the AG (OR 1.19, 95% CI 0.89–1.61) or GG (OR 2.16, 95% CI 1.56–3.00) genotypes. Similar results were obtained using dominant or recessive models, which produced respective ORs of 1.95 (95% CI 1.50–2.52, P < 0.001) and 1.52 (95% CI 1.15–2.00, P = 0.003). Using the dominant model (CC vs. CT+TT), the CC genotype of rs4822983 had a marginally significant protective effect compared to the CT and TT genotypes (OR 0.67, 95% CI 0.43–1.04, P = 0.06). Similar results were observed for the two SNPs (rs3805322 and rs4822983) by multivariate logistic

| Variable | Hangzhou case-control population | | Jinan case-control population | |
|----------|----------------------------------|------------------|------------------|------------------|
|          | (Discovery population)           | (Validation population) | (Validation population) | |
|          | Cases                          | Controls         | Cases           | Controls         | P† |
|          | No. (%)                        | No. (%)          | No. (%)         | No. (%)          | P† |
| **Age (years)** |                                   |                  |                  |                  |    |
| <62 (<56)  | 617 (50.4)                     | 534 (51.5)       | 540 (50.2)       | 550 (54.4)       | 0.711 |
| >62 (>56)  | 306 (49.6)                     | 259 (48.5)       | 269 (49.8)       | 251 (45.6)       | 0.167 |
| **Sex** |                                   |                  |                  |                  |    |
| Male      | 534 (86.5)                     | 462 (86.5)       | 428 (79.3)       | 453 (82.4)       | 0.988 |
| Female    | 83 (13.5)                      | 72 (13.5)        | 112 (53.6)       | 97 (17.6)        | 0.193 |
| **Smoking** |                                   |                  |                  |                  | <0.001 |
| Yes       | 425 (68.8)                     | NA               | 354 (65.5)       | 285 (51.8)       |    |
| No        | 192 (31.2)                     | NA               | 186 (34.4)       | 265 (48.2)       |    |
| **Drinking** |                                   |                  |                  |                  | 0.001 |
| Yes       | 411 (66.6)                     | NA               | 300 (55.6)       | 251 (45.6)       |    |
| No        | 206 (33.4)                     | NA               | 240 (44.4)       | 299 (54.4)       |    |

Abbreviations: NA, not available; NC, not calculated; ESCC, esophageal squamous cell carcinoma.

†Two-sided χ² test.

The median ages of the cases in the Hangzhou and Jinan populations were 62 and 56 years old, respectively.

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Table 2. Allele frequency distribution between the ESCC and control populations.

| SNP name | Gene | Location | Function | Major/ major allele | Major/ minor allele | Minor/ minor allele | No. of Major/ major alleles in cases | No. of Major/ minor alleles in cases | No. of Minor/ minor alleles in cases | No. of Major/ minor alleles in controls | No. of Minor/ minor alleles in controls | X² test (Hardy-Weinberg test for controls) | P value ((Hardy-Weinberg test for controls)) | P (Fisher’s test) |
|----------|------|----------|----------|---------------------|---------------------|---------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|----------------------------------------|-----------------|
| rs3805322 | ADH4 | 100056998 | Intron region | AA | AG | GG | 236 | 256 | 123 | 125 | 246 | 141 | 0.744 | 0.689 | 0.000 |
| rs4822983 | CHEK2 | 26719078 | Intron region | CC | CT | TT | 358 | 226 | 32 | 324 | 166 | 23 | 0.087 | 0.957 | 0.225 |
| rs10082466 | MBL2 | 52766862 | 3'-UTR | AA | AG | GG | 437 | 161 | 15 | 369 | 147 | 13 | 0.621 | 0.733 | 0.844 |
| rs1152620 | MALAT1 | 65489858 | N/A | AA | AG | GG | 188 | 120 | 303 | 149 | 112 | 267 | 161.733 | <0.001 | 0.6 |
| rs11548103 | S100A14 | 52766862 | 3'-UTR | GG | AG | AA | 49 | 286 | 278 | 58 | 231 | 239 | 0.038 | 0.981 | 0.198 |
| rs11716316 | ABI3BP | 100764451 | Intron region | AA | AC | CC | 186 | 138 | 286 | 150 | 117 | 262 | 152.489 | <0.001 | 0.6 |
| rs9327870 | ABI3BP | 10267853 | N/A | TT | TC | CC | 186 | 258 | 85 | 218 | 281 | 115 | 2.079 | 0.353 | 0.426 |
| rs12904 | EFNA1 | 155134221 | Near Gene-5 | AA | AG | GG | 451 | 152 | 11 | 393 | 125 | 11 | 0.082 | 0.959 | 0.862 |
| rs1321311 | CDKN1A | 36655123 | N/A | GG | GT | TT | 425 | 166 | 16 | 364 | 146 | 16 | 0.085 | 0.958 | 0.901 |
| rs17618244 | KLB | 39446909 | Missense | GG | AG | AA | 415 | 175 | 23 | 372 | 139 | 18 | 1.234 | 0.54 | 0.634 |
| rs2073498 | RASSF1A | 50332115 | Missense | CC | CA | AA | 530 | 81 | 4 | 461 | 64 | 5 | 2.614 | 0.271 | 0.74 |
| rs2114358 | mR-1206 | 12800833 | Intron region | TT | TC | CC | 314 | 181 | 32 | 339 | 238 | 37 | 0.315 | 0.854 | 0.194 |
| rs2385847 | DNH11 | 21544470 | Intron region | GG | GA | AA | 263 | 284 | 66 | 232 | 235 | 58 | 0.017 | 0.991 | 0.869 |
| rs351855 | *FGFR | 177093242 | Missense | TT | CT | CC | 402 | 5 | 191 | 334 | 14 | 146 | 430.686 | <0.001 | 0.035 |
| rs4072037 | MUC1 | 155192276 | Cds-synonymous | AA | AG | GG | 383 | 127 | 15 | 441 | 150 | 17 | 0.945 | 0.623 | 0.982 |
| rs550894 | mR-612 | 65444469 | ncRNA | GG | GT | TT | 342 | 238 | 31 | 282 | 208 | 35 | 0.163 | 0.922 | 0.466 |
| rs699517 | TYMS | 673016 | 3'-UTR | TT | CT | CC | 259 | 287 | 67 | 236 | 240 | 53 | 0.499 | 0.779 | 0.698 |
| rs8191664 | NEIL2 | 11786044 | Missense | GG | GT | TT | 400 | 191 | 22 | 355 | 148 | 25 | 3.382 | 0.184 | 0.431 |

Abbreviations: ESCC, esophageal squamous cell carcinoma; SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

* Because the number of heterozygous alleles was too small, the P values for comparisons of major/major allele vs. major/minor allele vs. minor/minor allele were not sufficiently persuasive. The comparisons of TT+CT versus CC or TT versus CT+CC were not significant. Thus, we did not further evaluate this SNP.

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regression analysis, which was adjusted for environmental factors such as smoking and drinking (Table 3).

The association of ESCC risk with these two SNPs (rs3805322 and rs4822983) was further validated in an independent case-control set (Jinan). Individuals with the ADH4 rs3805322 AA or AG genotypes had ORs of 1.10 (95% CI = 0.81–1.49, P < 0.001) or 1.86 (95% CI = 1.33–2.59, P = 0.559), respectively, for developing ESCC compared with those with the GG genotype. CHEK2 rs4822983 CC carriers showed a marginally significantly decreased ESCC risk compared with those harboring the CT and TT genotypes in the Jinan population (95% CI = 0.61–1.01, P = 0.064) (Table 3). The results became more significant when the two population sets were combined (Table 3).

Because of the key roles of ADH4 in ethanol metabolism and of CHEK2 in DNA damage repair, these two SNPs were further examined by stratifying the subjects in the Jinan cohort by smoking status and alcohol drinking history (Table 4). Interestingly, we found no significant association between the ADH4 rs3805322 genotypes and ESCC risk among the subgroups of either smokers or non-smokers (Table 4). There was a significant association between ADH4 rs3805322 genotype and ESCC risk among alcohol drinkers (OR 2.59, 95% CI 1.76–3.84). However, there was no statistically significant association between the CHEK2 rs4822983

### Table 3. Genotype frequencies of rs3805322 (ADH4) and rs4822983 (CHEK2) among cases and controls and their association with ESCC risk.

| Genotype | Hangzhou case-control set | Jinan case-control set | All patients |
|----------|---------------------------|------------------------|--------------|
|          | Cases No. (%) | Controls No. (%) | OR¹ (95% CI) | P¹ | Cases No. (%) | Controls No. (%) | OR¹ (95% CI) | P¹ | Cases No. (%) | Controls No. (%) | OR¹ (95% CI) | P¹ |
| ADH4     |                |                       |              |     |                |                       |              |     |                |                       |              |     |
| rs3805322|                |                       |              |     |                |                       |              |     |                |                       |              |     |
| GG       | 123 (20.0) | 141 (27.5) | 1.00 (Reference) | <0.001 | 117 (21.7) | 150 (27.3) | 1.00 (Reference) | <0.001 | 240 (20.7) | 291 (27.4) | 1.00 (Reference) | <0.001 |
| AG       | 256 (41.6) | 246 (48.0) | 2.20 (1.57–3.02) | <0.001 | 220 (40.7) | 261 (47.5) | 1.86 (1.33–2.59) | <0.001 | 476 (41.2) | 507 (47.7) | 1.13 (0.92–1.40) | 0.248 |
| AA       | 236 (38.4) | 125 (24.4) | 1.18 (0.88–1.59) | 0.276 | 203 (37.6) | 139 (25.3) | 1.10 (0.81–1.49) | 0.559 | 476 (41.2) | 507 (47.7) | 1.13 (0.92–1.40) | 0.248 |
| CHEK2    |                |                       |              |     |                |                       |              |     |                |                       |              |     |
| rs4822983|                |                       |              |     |                |                       |              |     |                |                       |              |     |
| CC       | 358 (58.1) | 324 (63.2) | 1.00 (Reference) | 0.089 | 224 (41.5) | 350 (63.6) | 1.00 (Reference) | 0.064 | 582 (50.3) | 674 (63.4) | 1.00 (Reference) | 0.064 |
| CT+TT    | 258 (41.9) | 189 (36.8) | 0.81 (0.63–1.03) | 0.089 | 316 (58.5) | 200 (36.4) | 0.79 (0.61–1.01) | 0.064 | 574 (49.7) | 389 (36.6) | 0.80 (0.67–0.95) | 0.051 |

Abbreviations: ESCC, esophageal squamous cell carcinoma; SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

¹ Multivariate logistic regression was used to evaluate the data adjusted for numerous variables, including age, sex, and smoking and drinking status.

### Table 4. Risk of ESCC associated with the rs3805322 (ADH4) and rs4822983 (CHEK2) SNPs by smoking status and drinking history in the Jinan set.

| Variable | ADH4 rs3805322 | PInteraction ² | CHEK2 rs4822983 | PInteraction ² |
|----------|----------------|----------------|----------------|----------------|
| Smoking status | | | | |
| No | 112/169 | 74/96 | 0.86 (0.58–1.27) | 0.443 | 143/197 | 97/102 | 0.76 (0.54–1.09) | 0.132 |
| Yes | 204/181 | 104/150 | 0.78 (0.57–1.08) | 0.131 | 173/153 | 127/98 | 0.87 (0.62–1.23) | 0.434 |
| Drunking status | | | | |
| No | 87/90 | 153/209 | 1.32 (0.92–1.90) | 0.131 | 112/169 | 74/96 | 0.86 (0.58–1.27) | 0.443 |
| Yes | 118/49 | 184/202 | 2.59 (1.76–3.84) | <0.001 | 204/181 | 150/104 | 0.78 (0.57–1.08) | 0.131 |

Abbreviations: ESCC, esophageal squamous cell carcinoma; SNP, single nucleotide polymorphism; No., Number; OR, odds ratio; CI, confidence interval.

¹ Logistic regression was used to evaluate the data adjusted for numerous variables, including age, sex, and smoking and drinking status.

² The multiplicative interaction term was used to calculate P values for gene-environment interactions.

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genotype and ESCC risk. In addition, the gene-environment interactions between the two SNPs (ADH4 rs3805322 and CHEK2 rs4822983) and smoking or drinking were evaluated using SPSS. No evidence of interaction existed between the two SNPs and smoking or drinking in the Jinan cohort (Table 4).

**Discussion**

Alcohol drinking is one of the most important modifiable lifestyle factors affecting EC, and alcohol metabolism has been suggested to play a central role in esophageal carcinogenesis [19]. Alcohol dehydrogenase enzymes (ADHs), which oxidize alcohol to acetaldehyde, are the most important and representative alcohol-metabolizing enzymes [20].

ADH4 is a key member of the ADH family of proteins encoded by seven ADH genes, ADH1A, ADH1B, ADH1C, ADH5, ADH4, ADH6, and ADH7 [21]. ADH4 exhibits the highest catalytic efficiency in the human ADH family; it may account for as much as 40% of the total ethanol oxidation rate at intoxicating levels of alcohol. The association between EC and polymorphisms in some of these ADH genes, including rs1229984 (ADH1B), rs698 (ADH1C), rs17028973 (ADH7), and rs671 (ALDH2), has been investigated [8, 22–26].

In our study, we examined the association between 18 selected SNPs in 17 cancer-related genes and the risk of developing ESCC in two Chinese populations. We found that patients with the AA genotype of rs3805322 (ADH4) had a significantly increased risk of developing ESCC compared with those with the AG or GG genotypes in our discovery dataset. Then, we successfully validated this result in a validation dataset (Jinan). In a matched case-control study including 585 patients with upper aerodigestive tract cancer and 1,170 non-cancer outpatients, Oze et al.[11] determined that compared with other genotypes, the GG genotype of ADH4 rs3805322 was associated with an increased risk of upper aerodigestive tract cancer in per-allele, dominant, and recessive models. However, this previous study, which was conducted in a Japanese population, only included 265 EC patients. The results of our study, which included a total of 1155 ESCC patients and 1062 controls, are inconsistent with this report [11].

Among DNA repair genes, CHEK2 (also known as Chk2 or Cds1) is a checkpoint kinase and transducer of cellular responses to DNA damage [27, 28]. Increasing evidence suggests that CHEK2 plays an important role in DNA damage signaling networks. In the current study, we observed that the CC genotype of CHEK2 rs4822983 showed a marginally significantly decreased ESCC risk compared with the CT and TT genotypes. When we combined the two groups, the significance increased. The results of our study are consistent with those of a genome-wide association study (GWAS) conducted by Wu et al. [29]. However, there was no interaction between the two polymorphisms (ADH4 rs3805322 and CHEK2 rs4822983) and drinking and smoking in terms of ESCC susceptibility.

Potential limitations of this study should be considered. First, this was a moderately sized case-control study with a total of more than 1,000 cases. The statistical power may be limited because of the sample size. Thus, it is important that the observed associations are validated in a larger study. Second, this was a hospital-based study; therefore, selection bias may be unavoidable. Third, data on smoking and drinking status were unknown in the Hangzhou case-control set and were therefore not adjusted for in the logistic regression models. A population-based study is needed to further validate our findings.

**Conclusions**

The current study provides substantial evidence that genetic polymorphisms of rs3805322 in the ADH4 gene may be associated with an increased risk of developing ESCC in two Chinese Han populations. Polymorphisms in ADH4 rs3805322 influence susceptibility to ESCC in
different genetic models of allele-dose effects and recessive effects. Future studies to address the biological function of these polymorphisms in the development of ESCC are warranted.

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Author Contributions

Conceived and designed the experiments: WM MY. Performed the experiments: JW AZ XX. Analyzed the data: XX YF. Contributed reagents/materials/analysis tools: QS SMZ. Wrote the paper: XX. Collected specimens: SMZ.

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