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Detection of a new species of torque teno mini virus from the gingival epithelium of patients with periodontitis

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Abstract We describe a novel species of torque teno mini virus called TTMV-204, which was isolated from the gingival epithelium of patients with periodontitis and characterized using viral metagenomics. The sequence of the full genome is 2824 nt in length. Phylogenetic analysis and genetic analyses show classic *Betatorquevirus* species organization with less than 40% amino acid similarity in ORF1. The prevalence of TTMV-204 in the periodontitis patient population was 18.75% (15/80), which was higher than in periodontally healthy individuals (10.00%, 10/80). However, the difference of the TTMV-204 prevalence between two groups was not statistically significant ($p = 0.115$). Further investigation is required to determine whether this new virus is associated with inflammation.

Keywords Anellovirus · TTMV · Periodontitis · Viral metagenomics

Introduction

The first anellovirus genome was described in 1997. Three genera of the family *Anelloviridae* were identified in humans: *Alphatorquevirus* (also known as torque teno virus or TTV, including 29 species, TTV 1-29), *Betatorquevirus* (also known as torque teno mini virus or TTMV, including 18 species, TTMV 1-18), and *Gammatorquevirus* (also known as torque teno midi virus or TTMDV, including 15 species, TTMDV 1–15) [1–3]. Anellovirus genomes consist of small, single-stranded, circular, negative-sense DNA with a size of 30 nm [4, 5], and their virions lack envelopes. Genome lengths vary from 3.7 to 3.8 kb for TTV, from 2.8 to 2.9 for TTMV, and approximately 3.2 kb for

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TTMDV [6, 7]. The anellovirus genomes contain a short, GC-rich untranslated region and three open reading frames (ORFs) encoding up to six different proteins through alternative splicing [8, 9]. ORF1 encodes the largest protein (capsid) and also contains Rep motifs associated with viral DNA replication [10–13]. ORF1 also has an arginine-rich N-terminus with DNA binding activity, which is thought to assist packaging of the viral DNA [11]. The hypervariable regions in ORF1 may represent mechanisms of immune response avoidance and chronic infection [14].

Anelloviruses were mostly being found in animal species. New torque teno virus isolates have been identified at a high rate since its first report. TTVs were classified into five genetic groups due to their high genetic variability, with 50% nucleotide sequence divergence at least [7]. Mechanism of the extensive genomic variation remains unclear. Anelloviruses are prevalent worldwide in the general population [2]. Human anelloviruses, especially TTV, have been examined since their discovery for association with various diseases, including hepatitis [13, 15], cancer [16], respiratory diseases [17], and hematological [18] and autoimmune disorders [19], but no evidence has been observed [7]. Recent studies have observed anellovirus co-infections and interactions between TTVs and the host immune system [20, 21]. Immunodeficiency has been associated with increased anellovirus plasma loads [22–28]. Genotypes of different anellovirus genera can co-infect the same individual and often predominate in different tissues [29]. It remains possible that subsets of human anellovirus genotypes are associated with particular diseases [30]. The viral load, co-infections with multiple types or other agents, the immune status of the host, and host genetics likely influence anellovirus pathogenicity [31].

Studies have reported that human oral cavity may be a habitat for anellovirus, which has been detected in respiratory tract fluids [17, 21] and saliva [32]. Indeed, anellovirus titers are higher in saliva than in sera [33], and the same species can be detected in both the serum and saliva of the same person [32]. Periodontitis is one of the most prevalent oral diseases that lead to the early loss of teeth [34]. While the incidence of periodontitis is high, its etiology remains unclear.

Recent studies have discussed the presence of viruses in patients with periodontitis, which may provide a new possible etiology perspective for this disease [35]. Identification of all new anellovirus species is necessary to understand their communities and their correlation with periodontitis. Identifying a correlation between anellovirus species represents the first step towards understanding the epidemiology, immunology, pathogenesis, and other aspects of this virus family. In the present study, viral metagenomics was employed to characterize a new species of TTMV, called TTMV-204, isolated from patients with chronic periodontitis in China.

Materials and methods

Identification of a novel TTMV by viral metagenomics

Samples were collected from patients visiting the Ninth People's Hospital, School of Medicine, Shanghai Jiao Tong University, China. A total of 48 patients with severe periodontitis were included, and biopsy specimens were obtained from the periodontal pockets gingival epithelium and connective tissue facing the sulcus during the periodontal surgery. Clinical data of all participants, such as age, gender, bleeding on probing (BOP), pocket depth (PD), clinical attachment loss (CAL), plaque index (PLI), gingival index (GI), alveolar bone resorption on pantomography, were recorded. Ethics Committee of the Ninth People's Hospital, School of Medicine, Shanghai Jiao Tong University approved this study (No. 201406), and written informed consent forms were signed by all participants before inclusion in the study. This study was performed in accordance with the principles of the Declaration of Helsinki.

The supernatant (300 µl) of samples was collected after centrifugation (10 min, 13,000×g) and filtered through a filter (0.45 µm) [3]. Then the viral nucleic acids were extracted and viral nucleic acid libraries consisted of both DNA and RNA viral sequences were constructed [3] [36]. Libraries were then sequenced on the MiSeq platform. Trimmed sequences of each group were assembled into contigs by Sequencher (Gene Codes) software, with criterion of at least 95% identity over 35 bp to merge two fragments. Singlet sequences and assembled contigs were then compared to those in GenBank using BLASTx method [37].

Amplification of the full-length of the new TTMV

The full-length of new virus was acquired by nested inverse PCR on the basis of sequences obtained from MiSeq analysis. An overlapping PCR fragment including the remainder of the circular genome was then obtained and sequenced. Amplification process was performed with Takara LA Taq polymerase and GC buffer I (LA PCR Kit Ver.2.1, TaKaRa, Dalian, China) as follows: 94 °C for 3 min, followed by five cycles of 1 min at 94 °C, 1 min at 60 °C, and 3.5 min at 72 °C, and then 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 3.5 min, with an additional 1 s in every cycle of extension stage and a final extension step of 10 min at 72 °C [3]. Conditions of the second-round cycling were the same as the first round. After electrophoresis on 1% agarose gels containing ethidium bromide (0.5 g/ml), the PCR products were excised and purified with an AxyPrep DNA Gel Extraction Kit

(Axygene, Silicon Valley, USA) according to the manufacturer's instructions, and then sequenced. Cloning of the newly obtained TTMV sequence with the same primers as above was performed in all the 48 samples.

Phylogenetic analysis and sequence similarity analysis

The aligned anellovirus sequences were trimmed to match the human anelloviruses present in GenBank using ClustalW. MegAlign software (DNAStar Inc., Madison, WI, USA) was used to perform the sequence analyses. A phylogenetic tree was constructed by the neighbor-joining method with ORF1 nucleotide p distances and 1000 bootstrap replicates in the Molecular Evolutionary Genetics Analysis program (MEGA, version 4.0, USA) using the ORF1 alignment sequences [3, 7]. Bootstrap values are indicated at each branching point. MEGA program was used to conduct the A % similarity analysis.

Investigation of the association of the new virus with chronic periodontitis

We conducted a case–control study consisting of 80 subjects with periodontitis (39 men and 41 women) and 80 periodontal-healthy subjects (42 men and 38 women) to investigate the association of the new virus with chronic periodontitis. Inclusion standards and biopsy specimens in the periodontal group were consistent with the first part of Methods. Subjects without periodontal diseases (no gingival inflammation, no BOP, no CAL, and no periodontal pockets) were included in the control group. Biopsy specimens were the gingival epithelium and connective tissue facing the sulcus and were obtained from periodontally healthy sites during tooth extraction. Prevalence of the newly identified virus was investigated by a hemi-nested PCR assay, with a final reaction mixture volume of 50 µl consisting of 3 µl of extracted DNA, 10 pmol of primers, and 25 µl of PrimerSTAR Max Premix (TaKaRa, Dalian, China). The first-round amplification parameters were 3 min at 94 °C, followed by 30 s at 94 °C, 30 s at 55 °C, and 50 s at 72 °C for 35 cycles, with a 5-min final extension at 72 °C. Conditions of the second-round cycling were the same as above. Amplification products were checked by electrophoresis on 1% agarose gels and then cloned and sequenced. The detection frequency (%) was determined for new virus-positive subjects.

Statistical evaluation was carried out by the SPSS 20.0 package (ver. 20.0; IBM Corporation, Armonk, NY, USA). The Student's t tests were used to compare the continuously variable data between the case group and the control group. Chi-squared tests were used to compare categorical variable data between the two groups.

Results

Detection of a novel human anellovirus

Forty-eight patients with chronic periodontitis (24 men and 24 women) aged from 18 to 65 years were recruited for this study. The viral particles and their nucleic acids in each sample were enriched and then sequenced. Based on the best BLAST score ($E < 0.001$), a novel viral sequence (204 bp) identified as a human anellovirus was obtained and further analyzed. Its full genome was obtained via nested inverse PCR with target-specific primers. The original patient with the virus was a 58-year-old female with alveolar bone resorption and BOP. Her mean PD, CAL, PLI, and GI values were 7.49, 4.65, 2.77, and 2.58, respectively.

Full-length nucleotide sequences and genome organization

Based on the novel viral 204-bp sequence, primers (S1 5′-A*G*TCTGGTGGAACGGGCAAG-3′, A1 5′-G*A*ATTGCCCCTTGACTACGG-3′, S2 5′-CAAAACCCAACATACTCTCC-3′, A2 5′-TGACTACGGTGGTTTCACTC-3′) were designed for amplification by inverse nested PCR and Sanger sequencing. The genome of the virus showed the characteristic features of a TTMV, consisted of 2824 bp, and had three ORFs (Fig. 1). ORF1 was the largest open reading frame at 1941 nucleotides (nt271–2211) long, encoding a 646 amino acid protein. Similar to other human anelloviruses, the capsid protein was arginine-rich in its N-terminus [4, 11]. ORF2 was 270 bp (nt122–391) long and encoded an 89 amino acid protein. Partial overlap occurred between ORF2 and

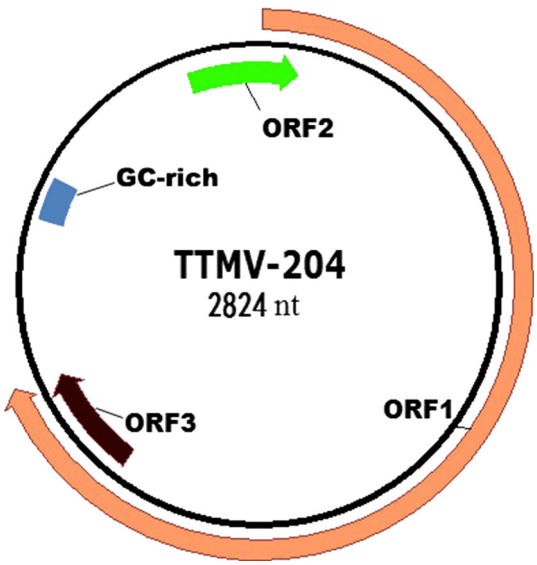

Fig. 1 The genome organization of TTMV-204. Annotations and illustrations were made using Vector NTI 10

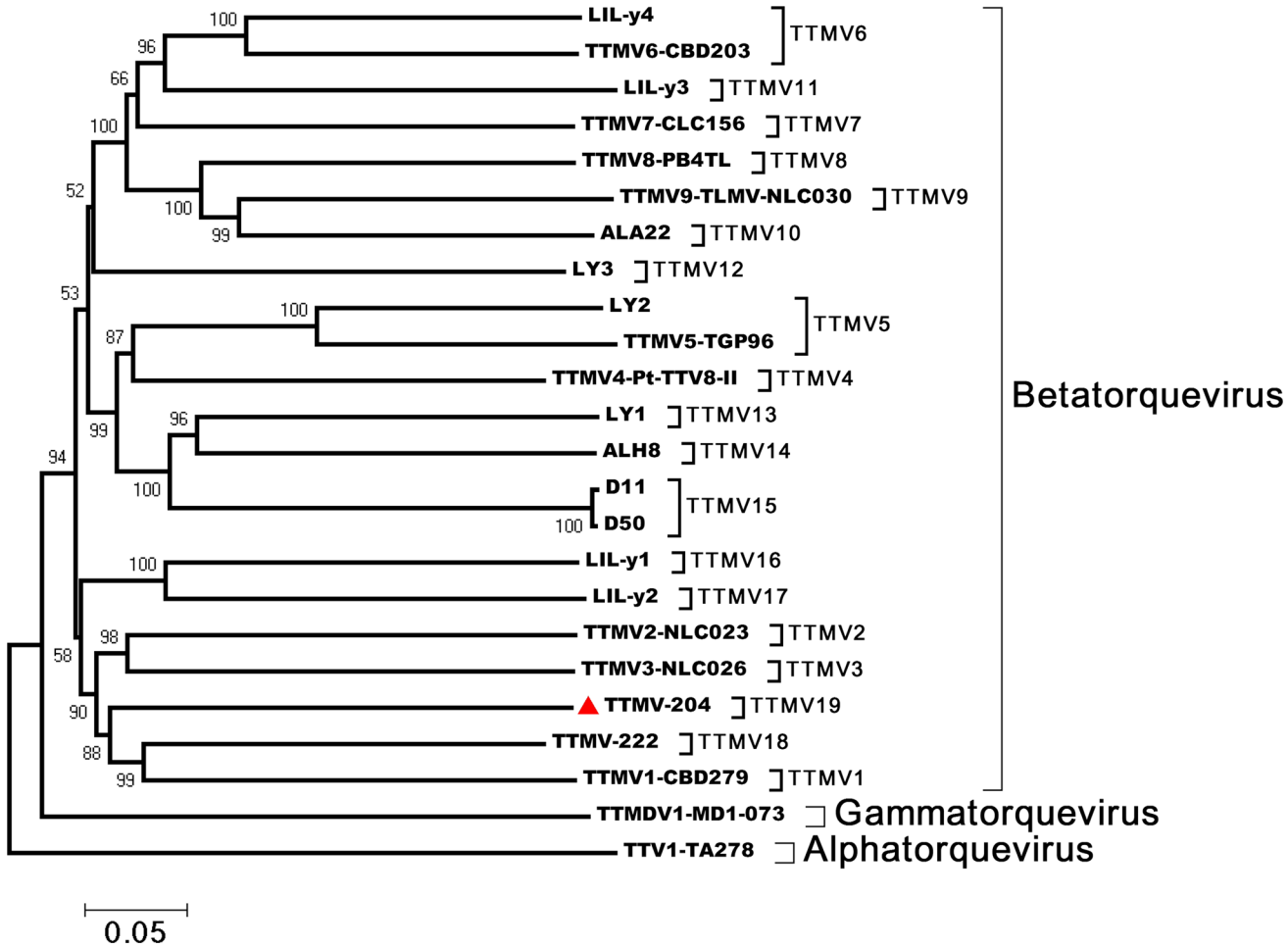

Fig. 2 ORF1 nucleotide phylogenetic tree constructed using the neighbor-joining method with nucleotide p distances and 1000 bootstrap replicates in the Molecular Evolutionary Genetics Analysis program (MEGA, version 4.0, USA). Bootstrap values are indicated at each branch point. The *solid triangles* indicate the novel TTMV species

ORF1 (nt271–391). ORF3 was 228 bp (nt1973–2200) long and encoded a 75 amino acid protein, which also overlapped with ORF1 (nt1972–2200). A GC-rich region was present in the non-coding part, as identified previously in other anelloviruses [8, 38]. These characteristics are consistent with the composition of other human TTMV strains previously reported [7, 39]. The sequence of the new viral genome isolate was deposited in GenBank (accession number: KU243129) and designated as TTMV-204. A total of three sequences containing the TTMV-204 were obtained after the cloning procedure. The sequences of the products shared high homologies with the primary sequence of TTMV-204 at a rate of more than 98.0%. Sequence analysis showed that the most common genetic diversity exhibited in ORF1.

Phylogenetic and homology analysis of TTMV-204

A phylogenetic tree (Fig. 2) was constructed with ORF1 of TTMV-204 and TTMV reference species [7]. TTMV-204 is related to TTMV1-CBD279, TTMV-222, TTMV2-NLC023, and TTMV3-NLC026, which belong to the TTMV family, genus *Betatorquevirus* (Fig. 2). Sequence analysis revealed that TTMV-204 has 57.6 and 57.1% nucleotide identity with the TTMV1-CBD279 (GenBank accession No. AB026931) and TTMV2-NLC026 strains (GenBank accession No. AB038630), respectively (Table 1). In ORF1, TTMV-204 shares the highest nucleotide similarity (57.5%) and protein identity (39.3%) with the TTMV-222 strain also derived from these periodontitis samples (GenBank accession No. KU041847) [3] (Table 1). The nucleotide and amino acid identities of TTMV-204 with reference strains of TTMV for ORF1, ORF2, ORF3, and the complete genome are listed in Table 1.

Prevalence investigation

One hundred sixty subjects were enrolled in the case–control study. No significant difference was found in

Table 1 Sequence similarity of TTMV-204 with other anellovirus strains

TTMV strain (GenBank accession No.)	Complete genome (%)	Tree Shrew TTMV-204 (KU243129)					
		Nucleotides (%)			Amino acids (%)		
		ORF1	ORF2	ORF3	ORF1	ORF2	ORF3
TTMV-222 (KU041847)	54.5	57.5	56.9	63.7	38.2	40.7	35.4
TTMV1-CBD279(AB026931)	57.6	55.8	53.0	56.6	38.7	36.8	47.2
TTMV2-NLC023(AB038629)	55.9	54.3	55.4	54.7	37.2	32.6	26.9
TTMV3-NLC026 (AB038630)	57.1	55.5	47.8	59.3	39.3	33.0	39.1
TTMV-LIL-y1 (EF538880)	54.2	53.8	49.2	52.3	31.4	45.5	26.8
TTMV_LY1 (JX134044)	53.9	50.0	47.2	52.2	19.9	24.4	33.3
TTMV_LY2 (JX134045)	53.7	47.6	48.6	48.4	31.3	39.3	27.5
TLMV-NLC030 (AB038631)	52.8	46.9	46.2	59.8	28.8	31.8	31.1
TLMV-CBD203 (AB026929)	53.7	48.8	51.0	56.1	30.2	36.6	39.7

Table 2 Comparison of periodontitis and control groups in age and clinical data

Group	Periodontitis group (mean ± SD)	Control group (mean ± SD)	*p*
Age	40.06 ± 12.13	38.53 ± 11.04	0.403
PD	8.44 ± 2.22	1.79 ± 1.12	<0.001
CAL	4.49 ± 0.75	0.12 ± 0.18	<0.001
GI	2.24 ± 0.36	0.17 ± 0.19	<0.001
PLI	2.57 ± 0.30	0.33 ± 0.27	<0.001

Obtained by student's *t* test (two groups)

$p < 0.05$

SD standard deviation, *PD* pocket depth, *CAL* clinical attachment loss, *GI* gingival index, *PLI* plaque index

gender and age between the two groups ($p = 0.874$ and 0.403, respectively). The mean PD, CAL, PLI, and GI values of the two groups were compared. These data are shown in Table 2. The mean PD, CAL, PLI, and GI values of the case group were significantly higher than the control group ($p < 0.001$). The grouping is reasonable.

Based on the amplified full-length sequence, primers (S1 5′-T*T*TACAACAACCTTACTGCCCAGAT-3′, A1 5′-G*A*AAAGGGTCCTGCCTCTGC-3′, A2 5′-CTTTGTCT GGGTTGTATCTTATTTCC-3′) were designed to measure the prevalence of the newly identified TTMV-204 in patients with chronic periodontitis and periodontally healthy participants. The 446-bp sequence was detected after the cloning procedure in 15 samples of 80 tested samples (18.75%) in the periodontitis group, and 8 samples of 80 samples (10.00%) were positive in the control group. Sequence analysis showed that the amplification products shared at least 95% nucleotide sequence homology with the targeted sequence of TTMV-204 (the nucleotide homology shown in Table S1 in the supplemental material online). The difference in the TTMV-204 prevalence between the two groups was not statistically significant ($p = 0.115$), though the TTMV-204 prevalence was higher in the periodontitis group than in the control group. There was no significant correlation between TTMV-204 and chronic periodontitis.

Discussion

In the present study, we characterized a novel human anellovirus in gingival tissue from patients with chronic periodontitis in China using viral metagenomics technology.

Chronic periodontitis is among the most prevalent disease worldwide, and it damages periodontal tissue and often results in early tooth loss [34]. Multiple infectious agents contribute to the pathogenesis of this disease, including periopathogenic bacteria, but do not account for all clinical symptoms of periodontal diseases. Viruses have also been implicated in the pathogenesis of periodontal diseases since the mid-1990s [40–42]. Individual periodontal lesions have been reported to harbor nucleic acids of herpes viruses, papilloma viruses, human T-lymphotropic virus-1 [43], hepatitis B virus [44], hepatitis C virus [45], human immunodeficiency virus (HIV) [46], and TTV [47]. Furthermore, a recent study showed that Epstein-Barr virus (EBV) enhances TTV replication [48].

EBV has been strongly suspected in the pathogenesis of periodontal disease [35, 49–52]. Our evidence has provided an additional perspective that anelloviruses contribute to periodontitis. However, the composition of the viral community in the periodontal environment and its association with periodontal diseases remain poorly understood. Recent metagenomics studies have discussed the possible role of bacteriophages in patients with periodontal disease [53, 54].

To investigate viruses in the microbial community of our sample set, we used an unbiased viral metagenomics method to characterize the periodontal virome [55–57]. TTMV-204 was detected by this method.

Phylogenetic analysis showed that the anellovirus genomes we described belong to Betatorqueviruses (TTMV). The phylogenetic tree analysis based on known TTMVs showed that a large number of genomes diverged from each other in ORF1 by over 42% at the nucleotide level and over 67% at the amino acid level [12, 58]. Overall, TTMV-204 was genetically closest to the TTMV1-CBD279 strain (GenBank accession No. AB026931) and the TTMV-222 strain (GenBank accession No. KU041847). Based on its genetic distance relative to other TTMVs, we propose that TTMV-204 belongs to a new species of *Betatorquevirus*.

A detection frequency of 15 TTMV-204-positive subjects among 80 patients with periodontitis was determined, and the prevalence of TTMV-204 was 18.75% (15/80) in chronic periodontitis patients. The TTMV-204-positive prevalence was 10.00% (8/80) in periodontally healthy subjects.

No significant association was observed between TTMV-204 and chronic periodontitis. The reported prevalence of TTMV was significantly different among geographical regions. Prevalence data from Brazil [59], Russia [60], Japan [61], and Pakistan [62] vary from 5 to 90%. Variations between these studies may result from the type of species, methods of detection, and design of PCR primers. Further investigation is required by expanding the sample size to determine whether TTMV-204 may be associated with chronic periodontitis or not.

In a former study [3], we have reported another anellovirus named TTMV-222 (GenBank: KU041847), which was obtained from the same 48 samples. It showed significant differences with the newly found TTMV-204. In the structure, these two anelloviruses share 42.5, 43.1, 36.3% genetic diversity in ORF1, ORF2, ORF3, respectively. In phylogenetic analysis, they generated independently evolutionary direction and were in different branches. In the taxonomy, TTMV-222 and TTMV-204 belong to a same genus *Betatorquevirus*, which provided a perspective that TTMVs may be widely exist in the human gingival epithelium. In the prevalence investigation, TTMV-222 showed significant association with chronic periodontitis, while TTMV-204 did not. It was considered that only certain species of human anelloviruses may be particularly pathogenic [7]. So more species of anelloviruses and their association with periodontal diseases require further evaluation.

Conclusions

The entire genome of TTMV-204 acquiring from periodontal pockets of patients with chronic periodontitis was obtained, and phylogenetic analysis determined that it was a new TTMV species. The epidemiological status of this new virus and its contribution to periodontitis require further investigation. The effectiveness of viral metagenomics for the genetic characterization of viruses in the periodontal environment was demonstrated, providing new evidence implicating viruses in periodontal diseases.

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Author contributions X.P.F and T.L.S designed the study. X.T.D and E.D completed the data analysis and statistics. Y.Z and F.L completed the sample collection and the amplifications of the newly discovered full-length human anellovirus. Y.Z, F.L, and X.C completed the epidemiological investigation. All of the authors have read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Ethics statement Ethics Committee of the Ninth People's Hospital, School of Medicine, Shanghai Jiao Tong University approved this study (No. 201406).

Informed consent Informed consent was obtained from all individual participants included in the study.

Data availability All data generated or analyzed during this study are included in this published article.

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