The Innovation

Report

Phylogenomic tracing of asymptomatic transmission in a COVID-19 outbreak

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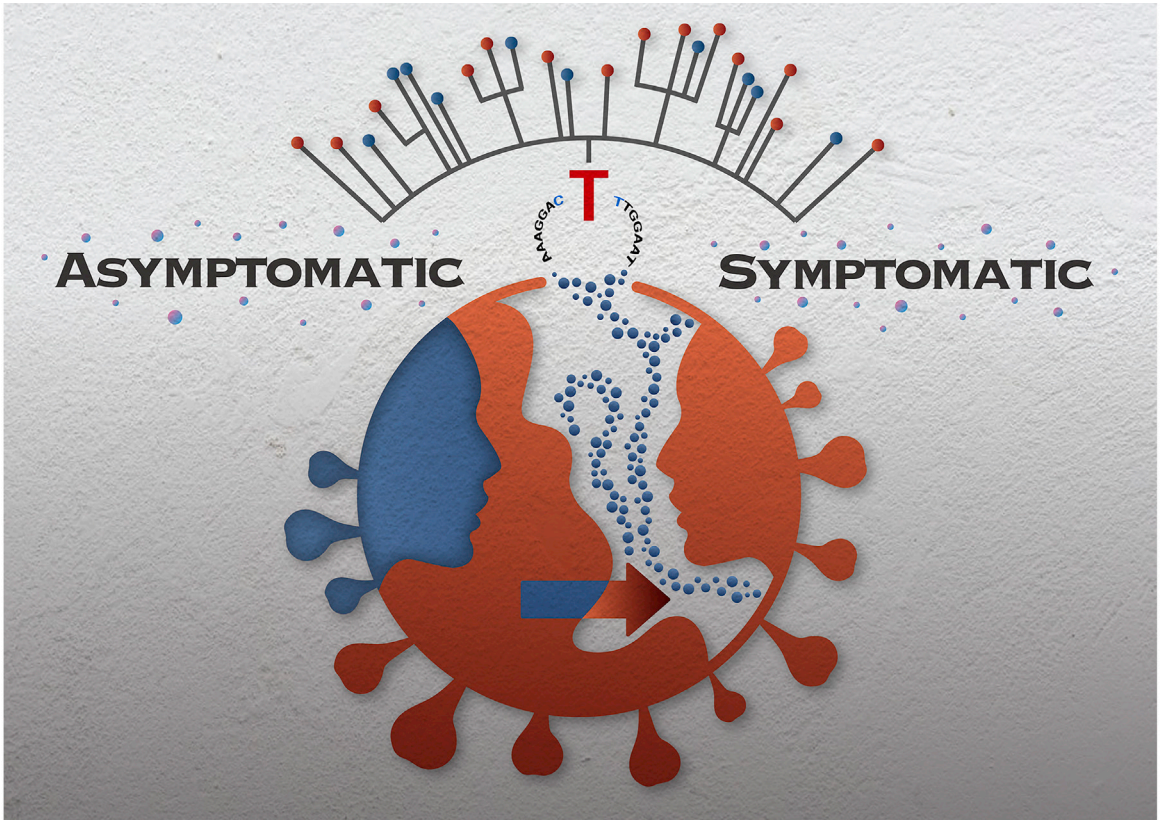
Graphical abstract



Public summary

- The silent transmission of SARS-CoV-2 poses a threat to public health
- The novel MINERVA sequencing technology reveals the accumulation of viral mutations during the asymptomatic transmission of SARS-CoV-2
- Close contacts of asymptomatic COVID-19 cases could develop into symptomatic patients
- Phylogenomic tracing could efficiently support the epidemic investigations of the COVID-19 outbreak

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SARS-CoV-2 has caused over 100 million deaths and continues to spread rapidly around the world. Asymptomatic transmission of SARS-CoV-2 is the Achilles' heel of COVID-19 public health control measures. Phylogenomic data on SARS-CoV-2 could provide more direct information about asymptomatic transmission. In this study, using a novel MINERVA sequencing technology, we traced asymptomatic transmission of COVID-19 patients in Beijing, China. One hundred and seventy-eight close contacts were quarantined, and 14 COVID-19 patients were laboratory confirmed by RT-PCR. We provide direct phylogenomic evidence of asymptomatic transmission by constructing the median joining network in the cluster. These data could help us to determine whether the current symptom-based screening should cover asymptomatic persons.

KEYWORDS: SARS-CoV-2; COVID-19; asymptomatic transmission; outbreak; phylogenomic

INTRODUCTION

By January 31, 2021, SARS-CoV-2 had infected over 100 million persons and led to more than 2 million deaths worldwide,[1] posing unprecedented challenges for population health, governments, and public health agencies. The threat of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) derives from not only its pathogenicity, but also its high transmissibility.[2–4] SARS-CoV-2 can be spread in communities by direct contact, respiratory droplets and airborne transmission, and through indirect contact with contaminated objects, especially those under freezing conditions.[5–8]

Asymptomatic persons seem to play a major role in SARS-CoV-2 transmission.[9–11] Asymptomatic COVID-19 patients include pre-symptomatic individuals and those who will never develop symptoms (asymptomatic carriers).[12] Unlike patients with SARS and Middle East respiratory syndrome (MERS), pre-symptomatic COVID-19 patients have a high viral load[13,14] and transmission capacity (accounting for an estimated 48% and 62% of cases in Singapore and in China, respectively).[10,15] On the contrary, only limited epidemiological investigations have suggested transmission from asymptomatic carriers.[16–20] Associated surveys based on cross-sectional design could not discriminate pre-symptomatic patients and asymptomatic carriers. In addition, epidemiological investigations cannot strictly rule out possibilities of infection by contaminated objects and aerosols in the environment, especially in endemic areas. Therefore, molecular evidence is urgently needed to elucidate the transmission chains containing asymptomatic carriers on the rapid epidemic.

Here, we traced a COVID-19 outbreak cluster in an apartment building in Beijing, China. From the 178 close contacts of the clinical index patient (P1), the other 13 COVID-19 patients were laboratory confirmed by RT-PCR, of whom five were asymptomatic during the quarantine and hospitalization. Using the novel sequencing strategy, MINERVA, which was driven by the urgency of the situation, providing more sensitive and deep sequencing of viral sequencing in clinical specimens with rather low viral loads,[21,22] we succeeded in obtaining all viral genomes from the 14 cases. Their genomes were tightly clustered into two clades, and provided a new insight into asymptomatic transmissions of SARS-CoV-2 with solid phylogenomic evidences.

RESULTS

The clinical index patient (P1) of the outbreak, a 58-year-old female, came back from Hebei Province on February 6, 2020, and was released from home quarantine on February 13. She began to experience symptoms of a sore throat on February 18 and a fever 3 days later. On February 24, qRT-PCR testing of pharyngeal swabs was positive for SARS-CoV-2 RNA.

On February 25, all close contacts (n = 178), including all the residents and staff in the apartment building, were identified by the Beijing Center of Diseases Prevention and Control, and were isolated for 14 days. Among them, 13 individuals (3 females and 10 males) were diagnosed with COVID-19 by clinical examination and qRT-PCR testing (Figure 1), and then hospitalized. All patients were diagnosed as mild pneumonia based on chest computed tomography scan (Table 1; Figure S2; supplemental information),[23] and were tested positive for the SARS-CoV-2 sera/plasma antibodies before discharge. Among 14 cases involved in this outbreak, five patients had developed mild respiratory symptoms, including a sore throat, cough, and stuffy nose before isolation (Figure 2; Tables 1 and S5); four patients developed COVID-19 symptoms during isolation and hospitalization; while the other five cases remained asymptomatic until discharged (Figure 2; Tables 1 and S5), accounting for 35.7% in the outbreak cluster. Of note, no patient with fever, except for P1, was observed before isolation. Detailed clinical information of the patients is listed in Table 1 and in the supplemental information (Tables S5 and S6). Of note, the viral shedding duration for the asymptomatic carriers was 11–52 days (median: 33 days), and 14–37 days for the symptomatic cases (median: 23 days, Figure 2; Table 1).

We retrospectively investigated the contact history. Six cases (P2–P5, P10, and P14) had no travel history. Eight cases (P1, P6–P9, and P11–P13) left Beijing before January 24, 2020, and returned to Beijing between January 29 and February 13 (Table 1). All 14 cases lived or worked in the building, and shared public spaces, including elevators and cafeteria. Despite spread of SARS-CoV-2, they only took limited self-protection measures. In addition, P1–P2, P6–P9, P10–P12, and P13–P14 shared apartments (Figure 1). P7 and P11 and P6 and P12 were close friends (Figure 1). Except for P2 and P5, the other patients worked for the property company owning the building. Six cases (P1, P4, P6, P7, P9, and P13) had close contacts during daily work.

To explore the molecular evidence of SARS-CoV-2 transmission in the outbreak, we obtained clinical specimens from the patients (sputum or fecal sample) on the dates labeled in Figure 2, and carried out the next-generation sequencing with DNA/RNA-hybrid tagmentation strategy, MINERVA.[22] We achieved viral genome sequences from all 14 patients (Table S1). Phylogenomic analysis of 13 high-quality (coverage: ≥70%) viral genomes was

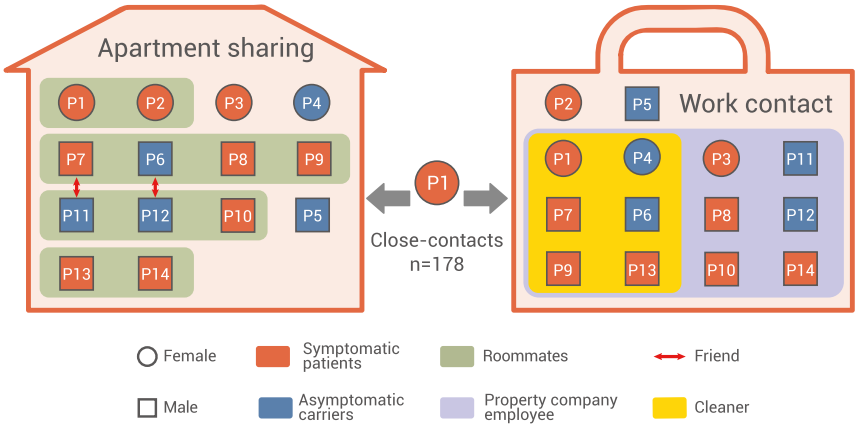

Figure. 1. Epidemic investigations of the patients The first discovered female patient with clinical respiratory symptom is marked P1 (middle). Fourteen confirmed COVID-19 patients (4 females and 10 males) were screened out from 178 close contacts by quarantine and qRT-PCR test of SARS-CoV-2 RNA during isolation. Patients living in the same room of the apartment building are labeled with a green background, and close friends except for roommates are connected with red double-headed arrows (left panel). Employees of the property company for the apartment building are labeled with a violet background, and cleaners in the company are labeled with a yellow background (right panel).

performed together with 72 strains circulating in Beijing during the same period, including 33 public viral genomes (from global initiative on sharing all influenza data [GISAID]) and 39 viral genomes from our center (Tables S3 and S4). Twelve viral genomes from this outbreak were tightly clustered into two clades with bootstrap values of at least 77 (clade I, P4–P7, P10–P12; clade II, P1–P3, P8, P13; Figure 3), and the viral genome of P9 was assigned as the closely related outgroup of clade I. We only obtained limited sequencing data (coverage: ~52%) from P14. However, given the results of two virus strains transmitted in the apartment, the genomic data from P14 supported its phylogenomic position located in clade II (results reported in the supplemental information; Figure S3; Table S7).

Since the viral genomes from all the five asymptomatic patients were assigned on clade I, we focused on the complete-genome diversity of clade I. Accordingly, the genome from P9 was identical to the consensus genome in Beijing at the same period (Figures 4A and S1), and was selected as the closely related outgroup (Figure 3B and 4B). We identified five reliable consensus variants on clade I, including two non-synonymous variants (C3429T and C18512T) and three synonymous variants (C337T, C6268T, and C17119T; Figures 4A and S3; Table S7). Within them, C18512T, C337T, and C17119T were inter-patient fixed variants. Among them, C18512T was observed in seven patients (P4–P7 and P10–P12), C337T in five patients (P4–P6, P10, and P12), and C17119T in two patients (P6 and P12, Figure 4). According to these inter-patient fixed variants, we constructed the median joining network with NETWORK (Figure 4B). Of note, among the five asymptomatic patients, P4 and P5 were identified to get the second fixed mutation (C337T), which induced the subsequent SARS-CoV-2 transmission involving two asymptomatic patients (P6 and P12) and a symptomatic patient (P10). The important variant loci related to asymptomatic transmission were covered by sequencing data (Table S4). In summary, the determinant mutations in the transmission chains at least suggested that the asymptomatic transmission was a reasonable deduction compatible with the available data, which was further supported by the epidemiological data (Figure 1; Table 1).

DISCUSSION

In the outbreak cluster, we observed that the asymptomatic carriers and minimally symptomatic patients (without fever) accounted for 35.7% and 28.6% of the cases (Table 1), respectively, which is consistent with previous studies.[16,17,24,25] It is worth noting that the four minimally symptomatic patients did not realize their SARS-CoV-2 infection and were missed by mandatory temperature checks. Therefore, when discussing prevention and control strategies for COVID-19, the minimally symptomatic carriers should be managed the same as asymptomatic cases.[17] Of note, all the patients in the outbreak developed pneumonia and produced immunoglobulin G against SARS-CoV-2 (Table 1; Figure S2), suggesting complete inflammatory immune response and transmission risk even in asymptomatic carriers.[26]

Here, we provide direct phylogenomic evidence for the asymptomatic transmission of SARS-CoV-2. The viral genomes in this outbreak were mainly tightly clustered into two clades, of which all genomes from the five asymptomatic cases were assigned to clade I (Figure 3). Only one mutation C18512T (p.6083P>L, Nsp14) was identified in clade I as non-synonymous mutation of three fixed SNPs, which were lack of functional data. In addition, this variant was not reported during the same period. Nsp14 is important for proofreading and replication of SARS-CoV-2[27,28]; however, more data are needed to investigate whether the mutation might cause more asymptomatic cases.

By tracing the virus mutation accumulation during the apartment outbreak, we found that two inter-patient fixed mutations (C337T and C17119T) were acquired in asymptomatic carriers (P4/P5 and P6/P12) and subsequently transmitted to others (Figure 4B). The transmission between P4 and P5 or between P6 and P12 might be also possible. The gradual accumulation of the viral SNPs could be observed, largely due to the closed management of the apartment building and the return of these individuals to Beijing at different times (Table 1). Of note, P10 who developed COVID-19 symptoms on February 25 was infected by P4 or P5 (Figure 4B). This suggests that SARS-CoV-2 was transmitted from asymptomatic carriers to symptomatic patients, which has rarely been evaluated in the current transmission model of COVID-19.[2,11] It has been shown that the estimated R0 would dramatically increase when asymptomatic transmission is taken into consideration.[29,30] In addition, it is also worth noting the long viral shedding duration of the asymptomatic cases (P4 and P11, Figure 2). If future studies could confirm the transmission ability of asymptomatic carriers at later stages of infection, the risk of asymptomatic transmission would further increase.

In the circumstances of the lack of an efficient vaccine and specific antivirus medication, identifying and isolating patients is still considered as the most important strategy for containing SARS-CoV-2 transmission.[23,31] The silent transmission chain of this novel virus, mediated by large numbers of asymptotic SARS-CoV-2 carriers underlines the important roles of these individuals in viral transmission, and brings into question the current strategy of symptom-based screening.[9,11,12,17] Given the increased PCR testing capacity and development of novel detection methods in many countries, expanding the screening system to asymptomatic carriers becomes both necessary and feasible.[32–34] The novel MINERVA sequencing technique effectively supports phylogenomic tracing of SARS-CoV-2 transmission, and should be promoted in the epidemic investigations into the COVID-19 outbreak. Updating the prevention and control strategy for the close contacts, especially asymptomatic and pre-symptomatic patients, might be an important way to contain further transmission and control the spread of the disease.

MATERIALS AND METHODS

Sample and data

On February 24, 2020, we initially observed one patient (P1) who was admitted to the Capital Medical University, Beijing Ditan Hospital (Beijing, China) with a sore throat, fever, pulmonary infiltrates on chest radiographs, and laboratory-confirmed COVID-19.

Table 1. Summary of clinical features and laboratory results of the COVID-19 outbreak cluster

Patient	Sex	Age	Return date to Beijing	Classification (WHO)	Symptoms	Interval from onset to isolation (days)	Viral shedding duration (days)	IgG	Computed tomography presentation	T cell count (cells/ mm^3, median)	$CD4^+$ count (cells/ mm^3, median)	$CD8^+$ count (cells/ mm^3, median)	$CD4^+/CD8^+$ ratio (median)	NK cell count (cells/ mm^3, median)	B cell count (cells/ mm^3, median)
P1	F	58	Feb 6	mild	COVID-19 symptoms with fever	6	21	+	pneumonia, bilateral infiltration, with patchy shadows and ground-glass opacity	920	596	252	2.37	182	185
P2	F	48	–	mild	mild respiratory symptoms	0	37	+	pneumonia, bilateral infiltration, with patchy shadows and ground-glass opacity	1,187	832	447	1.86	896	214
P3	F	40	–	mild	mild respiratory symptoms	4	27	+	pneumonia, bilateral infiltration, with patchy shadows and ground-glass opacity	1,681	622	945	0.66	188	246
P4	F	51	–	mild	no symptom	–	49	+	pneumonia, with patchy shadows	1,315	733	476	1.54	239	358
P5	M	32	–	mild	no symptom	–	11	+	pneumonia, with ground-glass opacity	958	582	368	1.58	48	45
P6	M	58	Feb 13	mild	no symptom	–	23	+	pneumonia, with patchy shadows and ground-glass opacity	1,919	491	942	0.4	281	74
P7	M	51	Feb 6	mild	mild respiratory symptoms	−1	23	+	pneumonia, with patchy shadows and ground-glass opacity	1,521	711	747	0.96	450	146
P8	M	35	Feb 3	mild	COVID-19 symptoms with fever	−1	23	+	pneumonia, with ground-glass opacity	797	592	136	3.3	71	132
P9	M	53	Jan 29	mild	mild respiratory symptoms	17	33	+	pneumonia, bilateral infiltration, with patchy shadows and ground-glass opacity	2,602	1564	739	1.48	499	292
P10	M	33	–	mild	COVID-19 symptoms with fever	−1	37	+	pneumonia, with ground-glass opacity	845	572	249	2.29	184	161
P11	M	50	Feb 6	mild	no symptom	–	52	+	pneumonia, bilateral infiltration, with patchy shadows	788	397	291	1.37	192	91
P12	M	51	Feb 12	mild	no symptom	–	33	+	pneumonia, bilateral infiltration, with patchy shadows and ground-glass opacity	1,069	516	445	1.16	181	133
P13	M	30	Feb 2	mild	COVID-19 symptoms with fever	0	23	+	pneumonia, bilateral infiltration, with patchy shadows and ground-glass opacity	1,429	975	450	2.16	148	341
P14	M	21	–	mild	COVID-19 symptoms with fever	−2	14	+	pneumonia, bilateral infiltration, with patchy shadows	806	588	242	1.61	88	47

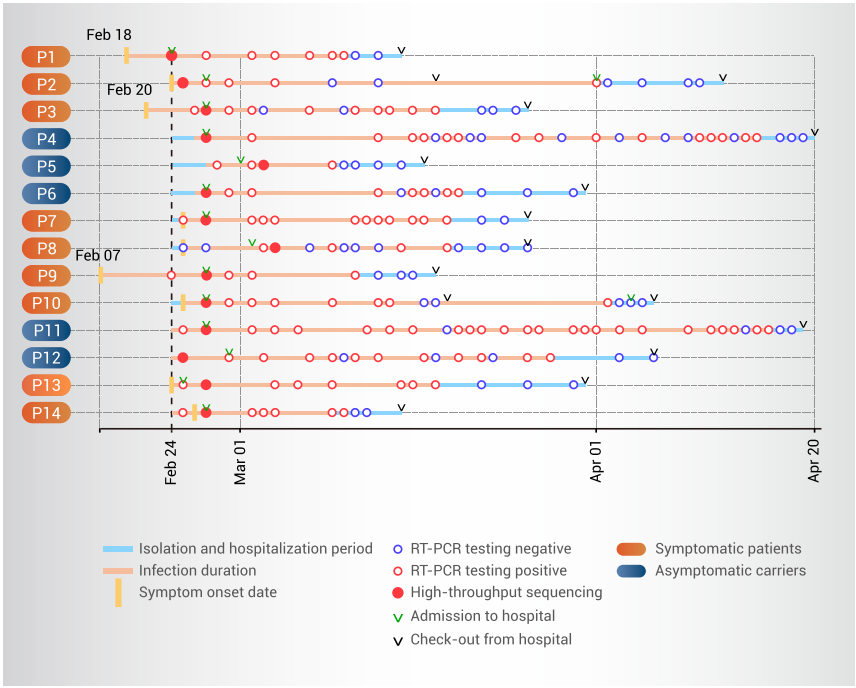

Figure. 2. Timeline of symptoms, clinical implementation, sampling, and laboratory test Patient numbers are shown for each case (left panel). The dates of clinical events, including symptom onset, admission to Beijing Ditan Hospital, checkout, sampling for qRT-PCR testing and sequencing, are labeled on the timeline (right panel). Red circles, the date with SARS-CoV-2 testing positive; blue circles, the date with SARS-CoV-2 testing negative.

The other 13 patients, screened from 178 close contacts, were also involved in the study. All the patients involved in the study were diagnosed based on Clinical Management of COVID-19 published by WHO.[35] An asymptomatic case is a person infected with SARS-CoV-2 who does not develop experienced symptoms that do not include radiological manifestation. We recorded and analyzed the history, physical findings, and hematological, biochemical, radiological, and microbiological investigation results. Samples from pharyngeal swabs, sputum, feces, and blood were collected according to the clinical guidelines. This study complied with the ethical principles for medical research involving human subjects according to the Declaration of Helsinki, and was approved by the Ethics Committee of Beijing Ditan Hospital, Capital Medical University (KT2020-006-01).

Viral RNA was extracted using the QIAamp Viral RNA Mini Kit according to the manufacturer's instructions, except that carrier RNA was omitted to facilitate downstream high-throughput sequencing analysis. The qRT-PCR assay targeted the open reading frame 1ab (ORF1ab) region and nucleoprotein (N) gene. A cycle threshold value less than or equal to 38 was interpreted as positive for SARS-CoV-2, according to Chinese national guidelines. All PCR results were collected from clinical data.

The MINERVA sequencing strategy is used to obtain the viral genome sequences of SARS-CoV-2 from all available specimens related to the outbreak (details in the first and second sections of the supplemental information).[22] In brief, after human rRNA removal and DNA digestion, the meta-transcriptome libraries further underwent the whole viral genome enrichment using the TargetSeq One Cov Kit (iGeneTech, Beijing, China). The final viral-enriched libraries were sequenced on an Illumina NextSeq500 in 2 × 75 bp pair-end mode. The approach generated 2.1 million (QRI: 2.95–12.59 million) viral reads per sample, respectively (Table S1). The sequencing data were deposited in the National Genomics Data Center, China National Center for Bioinformation (GSA: PRJCA002533, Table S2).

Data analysis

SNP identification. (1) Sequencing raw reads were trimmed to remove sequencing adaptors and low-quality bases (base quality < 20) with Cutadapt v.1.15.[36] (2) Clean reads were aligned to the reference genome of the SARS-CoV-2 (GenBank: NC_045512.2) using the Bowtie2 v.2.2.5[37] with default parameters. (3) Duplicate reads were removed with Picard Tools (v.1.141). (4) Samtools (v.1.10) "mpileup" was used to call SNPs using mpileup files as input with parameter -Q 20. (5) Each site was re-calculated and variants were screened using perl script with the following parameters: (ia) depth of alternate allele ≥ 5, (ii) alternate allele frequency ≥70%, and (iii) discarding the sites only supported by a single strand. The C337T variant in P4

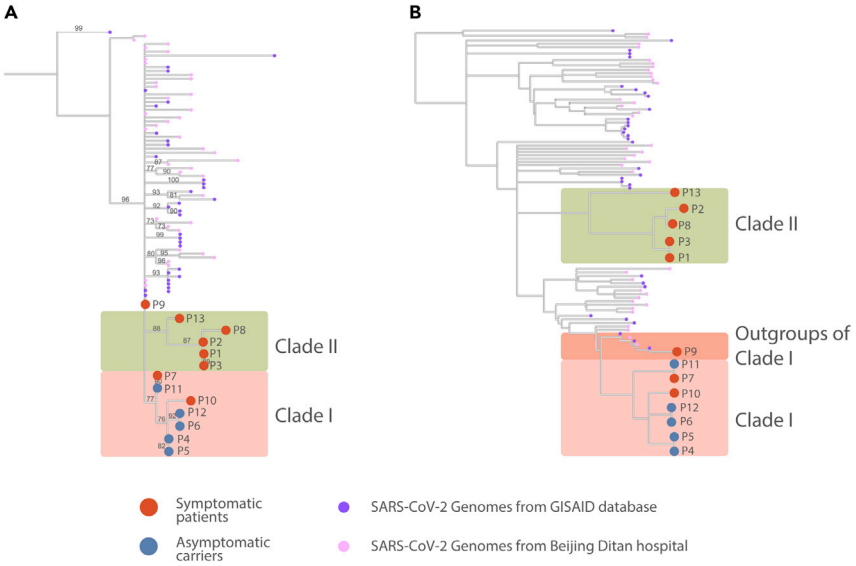

Figure 3. Phylogenomic analyses of viral genomes circulating in Beijing (A) The maximum likelihood tree constructed with viral genomes from the outbreak cluster and those circulating in Beijing at the end of February. Only branches with bootstrap values above 70 (labeled on branches) are shown.
(B) The time-resolved phylogenomic tree, the temporal structure and distribution of genomic clusters of which was properly modeled with a molecular clock approach. Orange spots, viral genomes of symptomatic COVID-19 patients in the outbreak; blue spots, viral genomes of asymptomatic carriers in the outbreak; pink spots, other genomes of patients from Beijing Ditan Hospital; purple spots, genomes from GISAID dataset.

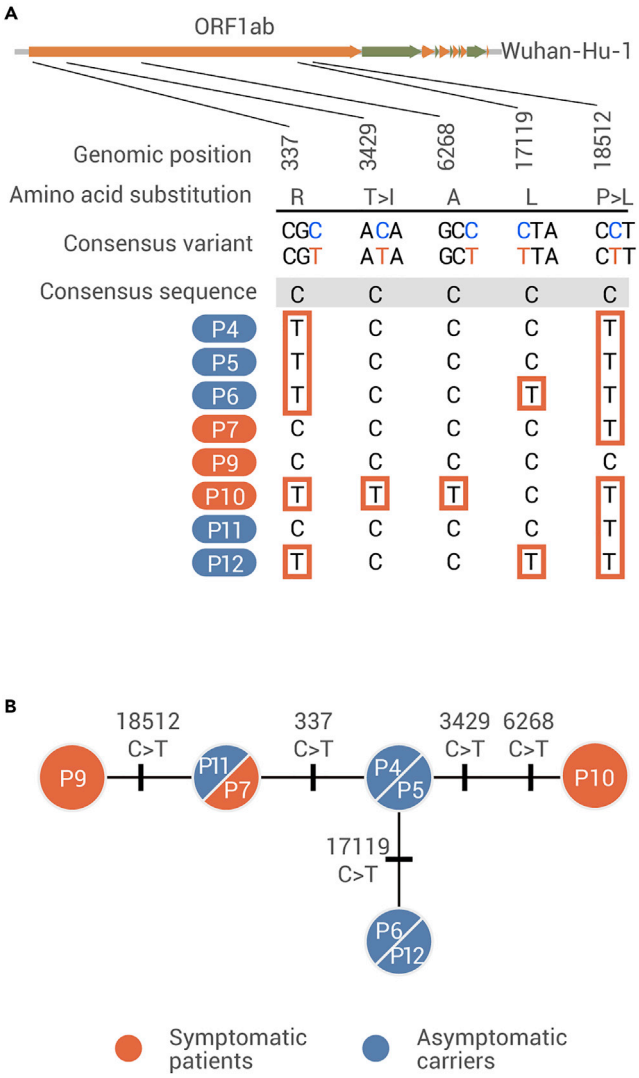

Figure 4. Median joining network of the outbreak cluster (A) The five SNPs identified in clade I. They are scaled on the SARS-CoV-2 consensus sequence, and substituted nucleotides in each patient are displayed at the bottom panel. (B) Proposed asymptomatic transmission network based on genetic distances. Each bold line on branches represents one SNP. The orange nodes, the patients with symptom; the blue nodes, the asymptomatic carriers.

were also considered as an SNP that was supported by sequencing reads (with 67% frequency) and validated by Sanger sequencing. (6) Consensus sequences were called using BCFtools based on reference sequence.

Phylogenomic analysis. We obtained 72 viral genomes with a sampling date before March 2, 2020. Among them, 33 genomes were from the GISAID (https://gisaid.org), 39 were from Beijing Ditan Hospital (GenBank: PRJNA667180) (Tables S3 and S4). The 33 genomes were selected from the GISAID database according to the following criteria: (1) sampled in Beijing, China; (2) with collection date before February 27, 2020; (3) completely assembled; and (4) with sequence length over 29,000 nt. We further discarded low-quality genomes with too much gaps or degenerate bases according to the assessment of National Genomics Data Center (https://bigd.big.ac.cn/)[38]. Consensus sequences were trimmed to 5′ and 3′ untranslated regions due to their poor quality. Multiple sequence alignment was conducted with parameters –auto –keeplength –addfragments using MAFFT v.7.45324.[39] The maximum likelihood tree was constructed using IQ-TREE v.1.6.12 with 1000 bootstrap replicates.[40] The substitution model GTR+F+R2 was selected based on Bayesian information criteria score. TreeTime v.0.7.6 was used for time-resolved phylogenomic analysis.[41] iTOL (itol.embl.de) was applied for displaying topology of phylogenomic tree.[42]

Transmission network construction. The nucleotide frequency of each genomic locus was calculated with the 85 viral genomes of circulating strains in Beijing, including 13 genomes (P1–P13) from the outbreak cluster and 72 local genomes mentioned above (Figure S1). A median joining network was constructed using NETWORK v.10.1.0.0 on the Fluxus Technology website (https://www.fluxus-engineering.com/).

A step-by-step procedure, including all the bioinformation commands and home-made scripts, was organized and uploaded in biocode (https://bigd.big.ac.cn/biocode/tools/BT007092/releases/1.0)

Measures of variables

The contact period is defined from the date when P1 returned to Beijing (February 6) to the date when the close contacts were isolated (February 24). The isolation and hospitalization periods were defined from the date of isolation to the date discharged from hospital of each patient, during which they were confined individually in a single room. The viral shedding duration was assessed from symptom onset to the SARS-CoV-2 test turning negative. As for the asymptomatic cases, the day before laboratory confirmation is defined as their onset time.

Data availability

We collected clinical and tracing data of patients from information managing system of the Beijing Ditan Hospital, as well as from publicly available data sources (published reports from Beijing public health agencies). All the information that we used is documented in the main text and in the supplemental information.

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AUTHOR CONTRIBUTIONS

J.Z., N.D., and Y.S. contributed to conceptualization, data collection, data analysis, interpretation of results, and writing of the manuscript. H.Z. and F.Z. contributed to conceptualization, interpretation of results, and writing of the manuscript. C.C., R.S., and L.L. contributed to data analysis and writing of the manuscript. Y.P., L.W., S.Y., Q.W., S.M., L.W., and F.Y. contributed to data collection and interpretation of the results.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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SUPPLEMENTAL INFORMATION

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