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Characterization of a Novel Reassortant Influenza A Virus (H2N2) from a Domestic Duck in Eastern China

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While H2N2 viruses have been sporadically isolated from wild and domestic birds, H2N2 viruses have not been detected among human populations since 1968. Should H2N2 viruses adapt to domestic poultry they may pose a risk of infection to people, as most anyone born after 1968 would likely be susceptible to their infection. We report the isolation of a novel influenza A virus (H2N2) cultured in 2013 from a healthy domestic duck at a live poultry market in Wuxi City, China. Sequence data revealed that the novel H2N2 virus was similar to Eurasian avian lineage avian influenza viruses, the virus had been circulating for ≥ two years among poultry, had an increase in α2,6 binding affinity, and was not highly pathogenic. Approximately 9% of 100 healthy chickens sampled from the same area had elevated antibodies against the H2 antigen. Fortunately, there was sparse serological evidence that the virus was infecting poultry workers or had adapted to infect other mammals. These findings suggest that a novel H2N2 virus has been circulating among domestic poultry in Wuxi City, China and has some has increased human receptor affinity. It seems wise to conduct better surveillance for novel influenza viruses at Chinese live bird markets.

Influenza virus remains one of the most common and important causes of human respiratory infections. Influenza virus outbreaks can result in high morbidity and mortality[1]. Outbreaks may be due to minor changes or antigenic drift (e.g. due to point mutations) or major genetic changes or antigenic shift (e.g. due to reassortment). Historically, pandemics have often been due to antigenic shift that corresponds to the emergence of novel zoonotic influenza viruses which have adapted to cause human-to-human transmission and disease[2,3]. Among the three types of influenza virus (A, B, and C), the influenza A viruses embody the greatest significant threat of host switching events, which is illustrated by the pandemic 2009 H1N1 virus (pH1N1), highly pathogenic H5N1 viruses, the recent emergence of human infection with novel avian origin influenza A H7N9 virus, and sporadic human infections with avian influenza A H10N8 virus in China[4–8]. Although H5, H7, and H10 subtype viruses have yet to cause efficient human-to-human transmission, their pandemic potential remains a serious public health threat.

Among the 18 currently recognized hemagglutinin (HA) subtypes[9–11], only H1, H2, and H3 viruses have recently been successfully established in humans. However, the best genetic data regarding human influenza only date back to the records of the influenza pandemic of 1918. Since then circulating influenza A viruses have had several antigenic shifts, resulting in the emergence of Asian/57 (H2N2, 1957-1968) and Hong Kong/68 (H3N2, 1968–1969) pandemic strains earlier in last century. H2N2 viruses have not circulated in humans since 1968 and persons born after that year have little or no immunity to H2 viruses[12]. Currently, H2 subtype influenza viruses are detected not only in avian species, also in swine. The oldest human H2 pandemic viruses are closely related to the currently circulating avian strains, suggesting that the pandemic H2N2 virus had an avian origin

and that antigenic change has been slow for H2 viruses[12]. Hence, H2 viruses remain a public health threat[13–15], and there is considerable risk that H2 viruses may reemerge to cause a pandemic similar to the 2009 H1N1 pandemic. It seems prudent then to study the mechanisms of introduction, adaptation, and transmission of avian H2N2 influenza viruses among terrestrial birds and to examine their potential for transmission to mammals.

Here we describe the characterization of a novel influenza A virus (H2N2) isolated in 2013 from a healthy domestic duck sampled in a live poultry market (LPM) in China. We investigated the pathogenicity and transmissibility of this multi-reassortant H2N2 isolate in mammalian hosts and the serological evidence of previous H2N2 virus infections among geographically-linked humans and live poultry.

Results

Identification of influenza virus among live poultry. The cloacal/environmental swabs from 325 adult chickens and ducks, environmental swabs of cages, and poultry drinking water from the LPMs were collected in Wuxi City during the period of June to December 2013. A total of 59 samples (18.2%) (Cycle threshold C_T values ranging from 25.4 to 36.9) were found to have molecular evidence of influenza A virus RNA by a real-time RT-PCR assay for the influenza matrix gene. Following the inoculation of these specimens into embryonated hens' eggs, five viruses were successfully cultured (all from specimens with a C_T value of <33). The insolates underwent full genome analysis by nucleotide sequencing and a BLAST search of the Influenza Sequence Database, which determined that one of isolates was a novel H2N2 influenza virus (GenBank sequence accession numbers KM100132 to KM100139). Additionally, another five isolates were determined to be known influenza viruses: one H5N1 virus isolate, two H5N8 virus isolates, and two H9N2 virus isolates (www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). The novel H2N2 virus was designated A/duck/Wuxi/2/2013(H2N2) (abbreviated as: A/duck/WX/2/13 (H2N2)).

Identification of evolutionary relationships and divergence time estimates. The phylogenies and time to most recent common ancestor (TMRCA) were computed for each gene segment of the novel H2N2 viruses and other influenza viruses. Clearly, all gene segments were nested within the influenza viruses from domestic or/and wild ducks, suggesting a pure avian origin of the novel H2N2 virus. When source locations were compared, remarkably all eight gene segments of the novel H2N2 viruses, HA, NA, NP, PA, PB1, PB2, NS, and M segments were very similar to the clades isolated from birds in east Asian (and east China in particular) in recent years (Fig. 1). Given the agreement in both the time and location (Fig. 2), we posit that the recent viruses circulating in ducks in East Asian around 2010 are the most likely donors of the six PB2, PB1, PA, NP, NA, and NS gene segments. However, we could not identify the immediate ancestors or ancestor-like viruses for the HA and M gene segments as the novel H2N2 virus' HA and M gene segment had considerable divergence from their genetically closest strains, this may reflect a novel virus or gaps in surveillance data.

We further sought to form a complete picture of the origins of the novel H2N2 virus by integrating the evolutionary analysis of its individual gene segments (Fig. 3). Although determining the precise evolutionary pathway of the genesis of the novel H2N2 is greatly hindered by the lack of surveillance data, we can conclude that the novel H2N2 virus emerged from continuously reassortant viruses circulating in Eurasian ducks. We first computed the TMRCA for individual gene segments. Table 1 shows proposed evolutionary events and their timing in the generation of the novel H2N2 virus. As illustrated in Table 1, the TMRCA for the six gene segments (NA, NP, PA, PB1, PB2, and NS) of the novel H2N2 virus ranged from January 2009 (NS) to October 2011 (NA), suggesting that the novel H2N2 virus has been circulating and left undetected for at least two years (10/2011-12/2013) in domestic and/or wild ducks. For HA, although the genetically closest virus A/environment/Guangdong/2/2009(H2N3) (abbreviated as GD (H2N3)) was identified from the existing influenza surveillance data, their TMRCA is September of 2005, showing a long period of time ($\sim$ 8 years) divergence between the novel H7N9 HA and GD(H2N3) HA. Similarly, there existed a long period of divergence time ($\sim$ 11 years) between the M of the novel H2N2 virus and that of its genetically closest virus A/duck/Eastern-China/29/2004(H6N2). Thus, we argue that the failure of detecting the immediate ancestors or ancestor-like viruses either from China or other countries for M and HA of the novel H2N2 virus is attributed to insufficient influenza samplings.

Molecular markers of host specificity and pathogenicity. To determine the host specificity and pathogenicity of novel H2N2 virus, we first analyzed the full-length of HA and PB2 gene sequences, focusing on the important residues in the receptor binding domain (S138A, T160A, G186V, Q226L, and G228S in HA gene, as well as E627K in PB2 of H3 numbering), which could contribute the adaption and virulence of avian influenza virus to human mammal host[16,17]. The HA of the novel H2N2 virus did not contain the multibasic cleavage site observed in highly pathogenic H5 or H7 AIVs, nor did the genome contain other major mammalian adaptations, such as the PB2 E627K substitution, which is known to alter host range and virulence. Infection of embryonated eggs was not pathogenic to the embryo. The receptor binding pocket residues have only one human-like amino acid at position A138 (H3 numbering), with the rest being avian-like with E190, G225, Q226, and G228, suggesting α2,3-linked sialic acid receptor binding preference. Other amino acids in the NS1 and matrix (M1; encoded by the M segment) proteins of the novel viruses are supposed be also associated with increased virulence. These amino acids are found in many avian H2N2 influenza viruses, and therefore, their significance for the biological properties of the novel influenza A(H2N2) viruses is currently unclear (table 2).

Receptor-binding properties of novel H2N2 virus. Molecular analysis of HA gene showed a human-like virus amino acid at position A138 (H3 numbering). As previous H2N2 viruses isolated from the wild birds have no human-like amino acid changes, we sought to characterize the receptor-binding properties of the novel H2N2 virus. Hence, we analyzed used solid-phase binding assays with A/Changchun/XD/2009(H1N1) and A/Chicken/Jilin/Hu-3/2006(H9N2) as control viruses to examine the receptor-binding properties of the novel H2N2 virus. The novel H2N2 virus was found to bind with both α2,6-linked sialyglycans (human receptor) and α2,3-linked sialyglycans (avian receptor); whereas, it will preferentially bind with the avian receptor (Fig. 4a) compared to the XD/H1N1 virus which had strong ability to bind to the α2,6-linked sialyglycans (Fig. 4b). In contrast, the avian H9N2 influenza virus bound only to the α2,3-linked sialyglycans (Fig. 4c).

Pathogenicity in chickens. To evaluate the pathogenicity of the novel H2N2 virus in chickens, we intravenously inoculated groups of ten 6-week-old SPF chickens. The viruses isolated were determined to be low pathogenic avian influenza by this inoculation as no clinical signs were observed by the animal care staff and all birds were scored as normal during the 10 day observation period. However, nine chickens developed elevated serum antibodies by the hemagglutination inhibition (HI) assay against the novel H2N2 virus on day 10 (6 chickens with HI titer $\geq$1 : 160, 1 with 1 : 40, and 2 with 1 : 20), while the two chickens inoculated with PBS were all negative by the HI assay.

Pathogenicity and transmissibility in mammals. To assess the potential risk of A/duck/WX/2/13 infection in mammals, we

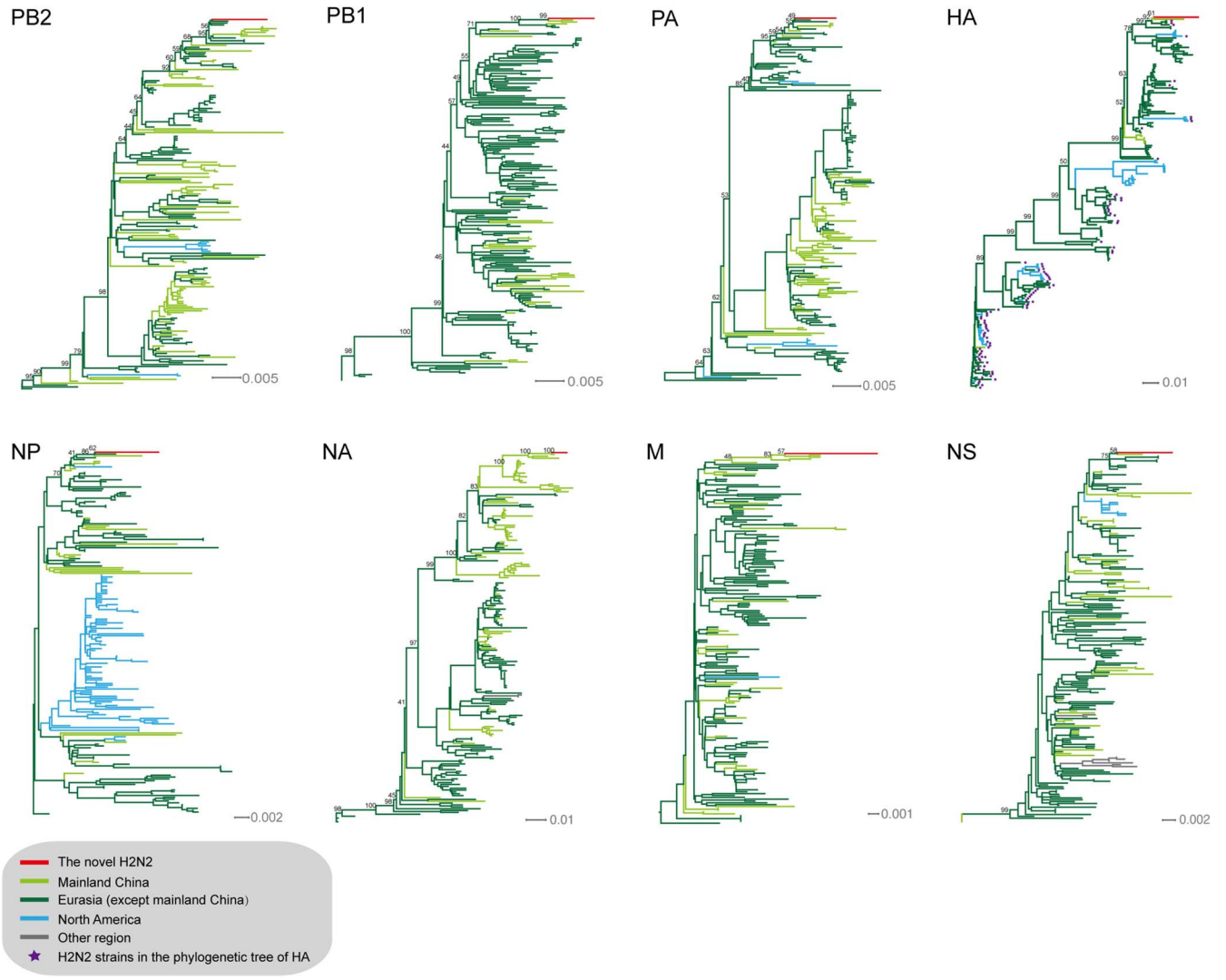

Figure 1 | Phylogenetic relationships of each gene of novel H2N2 strain and closely related viruses. All branch lengths are drawn to a scale of nucleotide substitutions per site. The novel H2N2 virus is highlighted in red. Detailed phylogenetic trees with virus designations can be found in Supplemental Figure 1.

administered the virus to BALB/c mice via intranasal routes. Intranasal administration of A/duck/WX/2/13 resulted in no viral replication, without detection of virus on day 3 and 5 post inoculation (p.i). The mice showed no clinical symptoms, weight loss, pathological changes, and had no detectable H2N2 virus serum antibodies by HI assay on day 14 p.i.

To assess the transmissibility of A/duck/WX/2/13 infection in mammals, we also administered the virus to guinea pigs via intranasal routes. Intranasal administration of A/duck/WX/2/13 resulted in no viral replication on day 2, 4, 6, 8, and 10 p.i, using nose wash specimens. The guinea pigs showed no clinical symptoms or weight loss; however, those inoculated guinea pigs had an elevated HI titer from 1 : 40 to 1 : 80 against the H2H2 virus by day 14 p.i. Furthermore, no HI titer was detected in any of the guinea pigs from the contact groups.

Serological analysis among human population and live poultry. Next we sought to assess the evidence of previous novel H2N2 infections among poultry workers and live poultry. Sera collected in the Wuxi City area during 2013 from 100 apparently healthy chickens/ducks and 1984 humans (including 560 poultry-exposed, 617 swine-exposed, and 817 study volunteers with no avian and swine-exposures) were analyzed using HI assays against the novel H2N2 virus. Nine percent of the poultry studied had HI titers $\geq$ 1 : 40 (Fig. 5a), among which one had a titer of 1 : 80 and two had an HI titer of 1 : 160. A total of 98 sera (4.9%) from human population had elevated antibodies against the novel H2N2 virus (HI titer $\geq$1 : 40). There was no statistically significant difference in antibody levels between the three exposure groups (Fig. 5b). Although, almost all of positive sera for the human population were from people at least 45 years of age (Fig. 5c), which likely indicates confounding by cross-reacting antibodies against human H2N2 pandemic virus.

Discussion

China has been implicated as the site of the origin of the 1957 and 1968 pandemic viruses[18]. Although, the H2N2 virus has not appeared to circulate among humans for several decades, avian H2N2 viruses are still circulating among birds and continue to pose a public health threat. China has been identified as an epicenter[19] for the generation of novel influenza virus strains; because, it has dense populations of humans and poultry[20] that often live in close proximity, some of the largest confined animal farm operations in the world, and a variety of enzootic swine and avian influenza viruses[21–23]. To better understand the epidemiology of zoonotic influenza viruses, we conducted a surveillance study of avian influenza virus in LPMs in China, and isolated a novel reassortant avian-origin H2N2 virus from a domestic

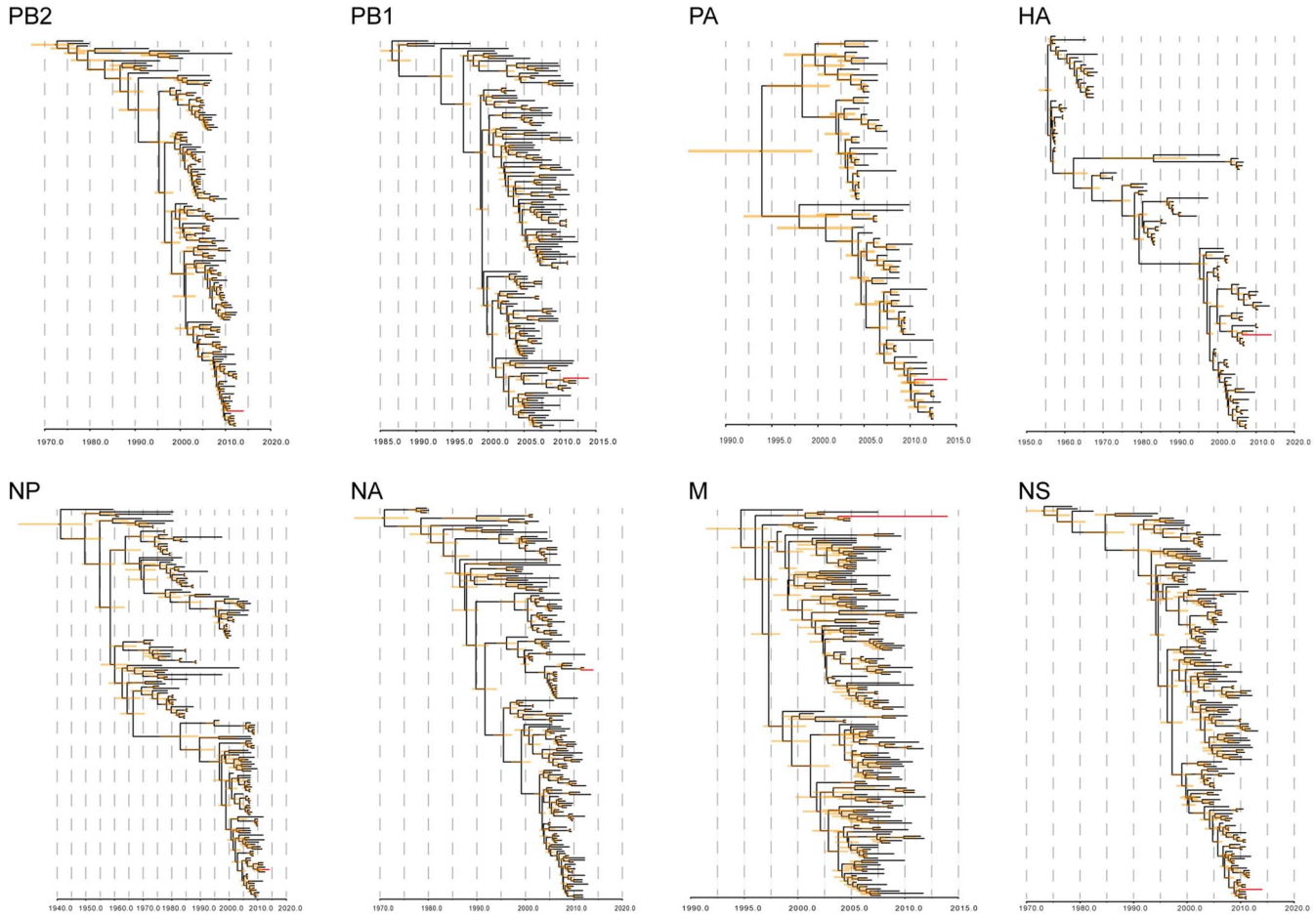

Figure 2 | Temporal reconstruction of the phylogenetic relationships of each gene segment. The novel H2N2 strain is highlighted by red. Internal nodes are reconstructed common ancestors with 95% highest posterior density (HPD) intervals on their dates given by the light red bars. Detailed temporal reconstruction of phylogenetic trees with virus designations can be found in Supplemental Figure 1.

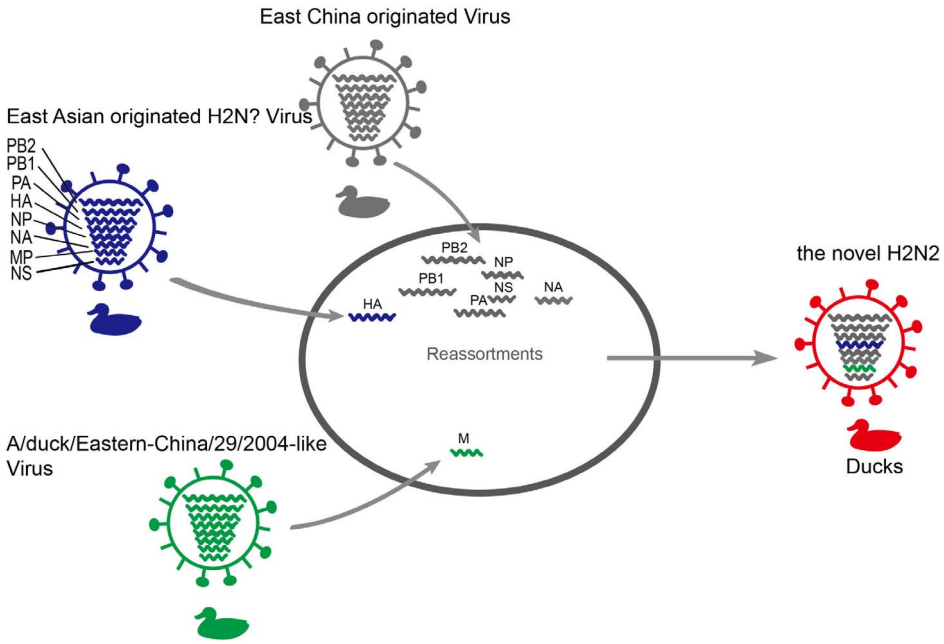

Figure 3 | Reassortment model for the origins of the novel H2N2. The colors of the novel H2N2 gene segments indicate their origins. The picture of duck was drawn by the author Mai-Juan Ma.

Table 1 | Time of most recent common ancestors (tMRCA) for the novel H2N2 virus. Observed closest strains for the H2N2 segments, time of divergence, and the mean evolutionary rate are listed

Gene	Divergence time	Observed closest strain	Mean evolutionary rate (10^{-3})
PB2	May 2010 (Dec 2009, Oct 2010)	A/mallard/Korea/1203/2010_H10N8	2.133 (1.849, 2.421)
PB1	Jul 2010 (Sep 2009, Mar 2011)	A/duck/Hunan/S11200/2012_H4N6	2.591 (2.344, 2.846)
PA	Jun 2010 (Apr 2009, Jul 2011)	A/duck/Vietnam/LBM185/2012_H6N6	1.864 (1.405, 2.351)
HA	May 2006 (Jan 2005, Aug 2007)	A/environment/Guangdong/2/2009_H2N3	3.859 (3.462, 4.278)
NP	Jun 2010 (Oct 2008, Sep 2011)	A/duck/Vietnam/LBM81/2012_H11N9	1.562 (1.305, 1.816)
NA	Jun 2011 (Jul 2010, Jan 2012)	A/duck/Jiangsu/m234/2012_H5N2	3.049 (2.697, 3.398)
M	Oct 2003 (May 2002, Sep 2004)	A/duck/Eastern-China/29/2004_H6N2	1.696 (1.400, 2.011)
NS	Sep 2009 (Nov 2008, Jun 2010)	A/duck/Nanjing/1102/2010_H4N8	2.357 (1.977, 2.773)

The mean evolutionary rate is represented as the number of substitutions per site per year. The values in parentheses represent the 95% highest posterior density (HPD) intervals.

duck in Wuxi City. To our knowledge, this is first time a H2N2 virus has been isolated from a domestic duck in mainland China.

According to data from NCBI[24], during the past decades, only a small number avian H2N2 viruses have been detected or isolated from wild birds or domestic poultry in North America, the Netherlands, Sweden, Japan, and Hong Kong. Based upon molecular and phylogenetic analyses, the H2N2 virus evaluated in our present study was a novel recombinant influenza virus strains, demonstrating divergence over time of PB1, PB2, PA, and NP gene segments. The most similar H2N2 strain was identified around 2010, suggesting that novel virus gene segments may be from the same influenza virus strain. Among the other three gene segments (HA, M, and NS) the most similar strains were identified long ago and hence these gene segments may represent independent recombination events. The novel H2N2 virus' NA gene segment is most similar with a H5N2 influenza virus strain isolated in 2012 in Jiangsu Province, which again suggests a recombinant origin of the novel H2N2 virus or this may support gaps in surveillance data due to insufficient influenza sampling between January 2009 and October 2011. However, it remains clear that in general the novel H2N2 virus is quite similar to the 1957 H2N2 human pandemic strain that disappeared in 1968.

The genetic characteristics of HA and PB2 gene segments are frequently associated with zoonotic transmission success or failure among avian influenza strains[16,17]. In avian H2 and H3 influenza viruses, HA receptor-binding-site residues corresponding to codon positions 138, 190, 194, 225, 226, and 228 (using the H3 numbering system) are highly conserved[17,25]. In human H2 and H3 viruses, leucine and serine substitutions at residues 226 and 228, respectively, have been shown to accompany their adaptation from avian to human hosts[26]. Our novel H2N2 virus demonstrated no such Q226L and G228L substitution in HA and no E627K substitutions in PB2, which is typical of AIVs (Table 2). However, position 138 of HA did have substitution, which could increase the binding affinity of human receptor. The receptor binding affinity assay revealed that the novel H2N2 virus had increased α2,6-binding compared with a previously isolated H2N2 avian virus. However, our experimental data suggested that the virus manifested only low replication in guinea pigs, and no replication occurred in direct or indirect guinea pig contacts or in BALB/c mice. This may suggest that the novel H2N2 virus currently has low risk of infection and transmission in mammals, and would have to further adapt to mammalian hosts to become a public health threat. It is interesting to note that Jones *et al.*[27] evaluated avian H2N2 influenza viruses and concluded that all H2N2 viruses they tested had almost no human receptor binding affinity using solid-phase enzyme-linked immunosorbent assays, whereas some H2N2 virus could replicate in DBA2/J mice and ferrets. It seems that receptor-binding changes are required for the successful adaptation of avian viruses to humans; however, this trait alone is not sufficient for human-to-human transmission. For example, the classical swine H1N1 influenza viruses have a preference for α2,6-linked glycans sialic acid viral receptors[28] and a number of self-limiting human transmission infections do occur; however, these viruses have not successfully established themselves in human populations. An examination of the other viral proteins shows that a number of substitutions are present when the novel H2N2 isolate was compared with other H2N2 influenza viruses isolated from swine or mallard ducks, although their significance is unclear.

We examined the pathogenicity of the virus in chickens and also looked for evidence of novel H2N2 virus transmission in geographically-linked poultry and human population. The results revealed that the novel H2N2 virus is a low-pathogenic avian influenza virus (LPAI), whereas 9% of examined poultry had elevated antibodies to the novel virus. However, the serological finding might be explained by antibodies against other H2 viruses such as H2N9[29], H2N7[30], and H2N3 which have also been rarely found to circulate among ducks in

Table 2 | Selected characteristic amino acids of the novel influenza A(H2N2) virus

Viral protein	Amino acid position	A/duck/WX/2/13	Comments
PB2	627	E	E627K: Mammalian host adaptation[42,43]
HA	138	A	S138A: Increased virus binding to human-type receptors[44]
	186	N	G186V: Increased virus binding to human-type receptors[45]
	190	E	E190D: Increased virus binding to human-type receptors[46]
	225	G	G225D: Increased virus binding to human-type receptors[46]
	226	Q	Q226L: Increased virus binding to human-type receptors[26]
	228	G	H2N2 Human: S[47]
NA	65-71	No deletion	Deletion of 65–71 increased virulence in mice[48]
M1	30	D	N30D: Increased virulence in mice (most influenza A viruses encode 30D)[49]
	174	R	K174K(R): Host marker of influenza A[44]
NS1	42	S	P42S: Increased virulence in mice (most avian influenza A viruses encode 42S)[50]
	97	E	D97E: Increased avian H5N1 viruses' virulence in mammals[47]
	127	N	TDRVA127N: Association with H3N2 virulence[47]

HA amino acid residues have H3 numbering; NA amino acid residues have N2 numbering.

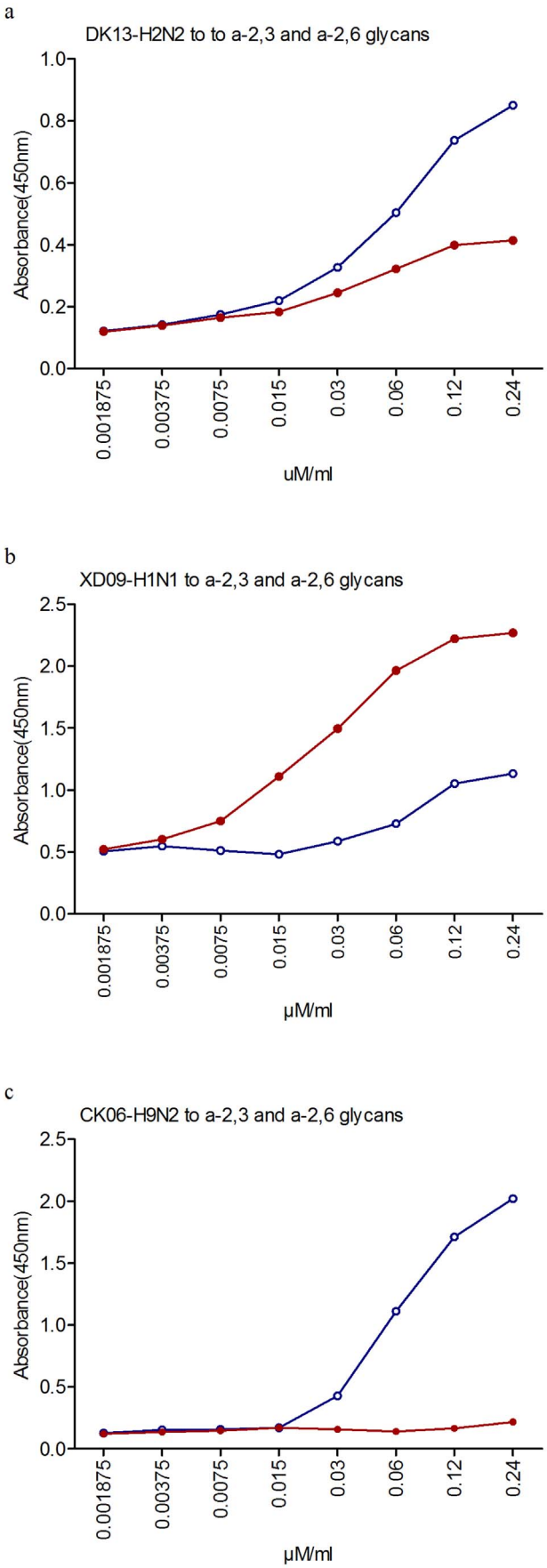

Figure 4 | Characterization of the receptor-binding properties of isolated H2N2 virus. Binding of virus to a2,3-linked (3′SL-PAA) or a2,6-linked (6′SL-PAA) sialylglycan receptors was determined by solid-phase binding assays. (a) DK13-H2N2 (A/duck/Wuxi/2/13 (H2N2)); (b) XD09-H1N1 (A/Changchun/XD/2009 (H1N1)); (c) CK06-H9N2 (A/Chicken/Jilin/Hu-3/2006(H9N2)). Blue line, binding to (3′SL-PAA); red line, binding to (6′SL-PAA).

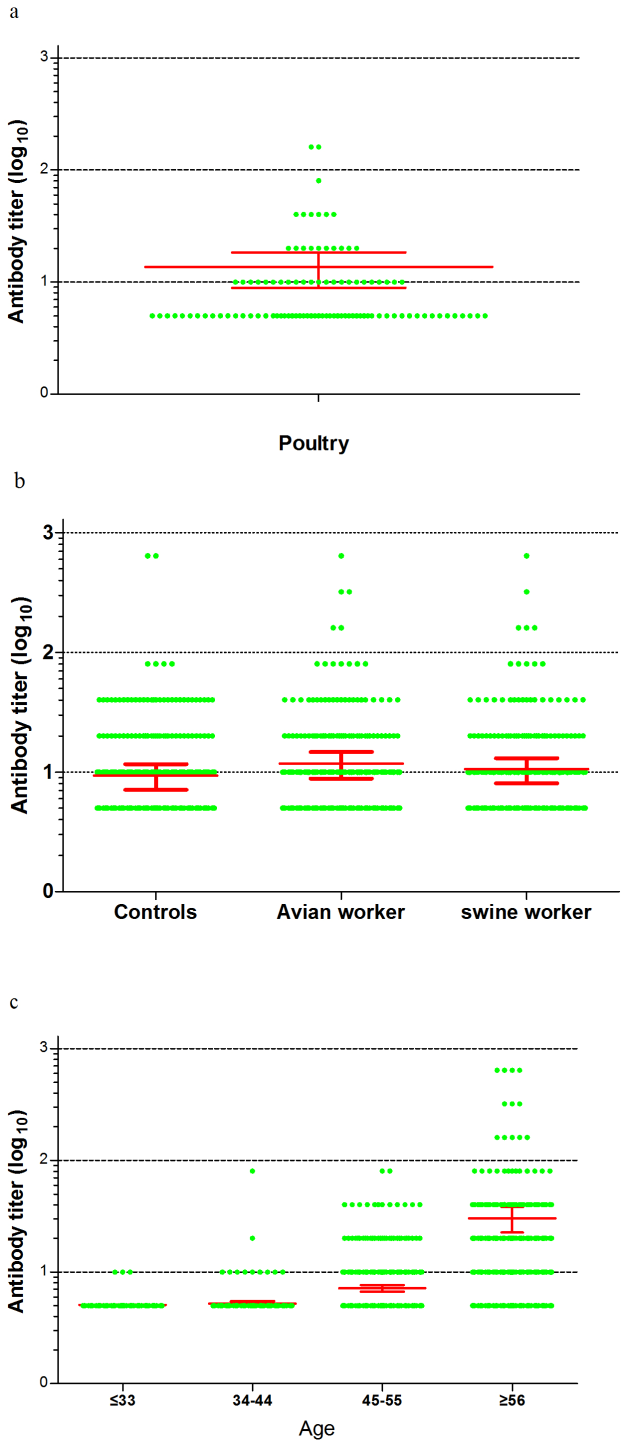

Figure 5 | Hemagglutination inhibition (HI) Antibody against H2N2 virus among human population and live poultry (mean antibody counts shown with 95% confidence intervals). (a) HI antibody of H2N2 virus among poultry. (b) HI antibody of H2N2 virus among general health population, poultry workers, and swine workers. (c) HI antibody of H2N2 virus among different age population.

Asia. No matter the H2 type, our data suggest that the H2 viruses continue to circulate within LPMs. Our study found that 4.9% of 1984 human participants had elevated antibody to the novel H2N2 virus, of which, people under age of 45 have little or no immunity, and resistance dramatically increased for those older than 45 (figure 5). This was also the case for the 2009 H1N1. The low mutation rate for H2N2 and evidence of waning human immunity make it likely that an H2N2 pandemic could arise from a zoonotic H2N2 strain[13,15,31].

Avian influenza viruses are often thought to be transmitted from wild birds to domestic birds and then to people. However, influenza viruses from wild birds seldom adapt to and replicate well within domestic birds[32], preventing people from being infected by the wild bird avian influenza virus strains. However, when a rare wild bird avian influenza adaption event occurs, such viruses may further adapt for transmission to humans. Several subtypes of avian influenza viruses found in poultry are capable of infecting humans, which include high-pathogenic avian influenza virus (H5N1, H7N2, and H7N7), low-pathogenic avian influenza virus (H9N2, H7N9, and H10N8), and potential other unknown avian influenza viruses[32].

In summary, while the immediate potential for this virus to infect humans and to be transmitted efficiently from human-to-human seems unlikely, several characteristics of the virus are quite troubling: the low viral evolution, the increased human receptor binding affinity, and the lack of human immunity for H2N2 virus suggest this and other H2 progeny viruses circulating in China may be a cryptic threat to man. Hence, it seems wise to increase surveillance for novel viruses among healthy poultry in China's LPMs. Such active surveillance and subsequent viral characterization of isolates seem extremely important in evaluating emerging viral threats, in following antiviral susceptibility, and in designing both poultry and human influenza vaccines, all of which are critical components in the public health response to influenza.

Methods

Ethics statement. The protocol of the study was conducted in accordance with guidelines of animal welfare of World Organization for Animal Health for animal experiments and Declaration of Helsinki for human experiments, and approved by Animal Welfare Ethics Review of Academy of Military Medical Sciences and Institutional of Review Board of Wuxi Center for Disease Control and Prevention.

Samples collection. We collected cloacal/environmental swabs from chickens and ducks in LPMs for avian influenza virus surveillance during the period July 2013 to December 2013 in Wuxi City of Jiangsu Province, China. Swabs were placed in transport medium, consisting of phosphate-buffered saline (PBS) containing 50% glycerol, penicillin (2,000 U/ml), gentamicin (250 μg/ml), polymixin B (2,000 U/ml), nystatin (500 U/ml), ofloxacin HCl (60 μg/ml), and sulfamethoxazole (200 μg/ml). Swab specimens were kept on ice for up to 4 h before their preservation −80°C.

RT-PCR and virus Isolation. The viral RNA of each swab sample were extracted using QIAamp MinElute Virus Spin Kit (Cat.No.57704, Qiagen) as directed by the manufacturer. All samples were tested by a one-step real-time RT-PCR assay targeting the influenza matrix genome segment[33], using a one-step RT-PCR kit (Cat. No. 56046, TaKaRa), on an Applied Biosystems 7500 real-time PCR system (Life Technologies, NY, USA). Then, 200 μl RT-PCR-positive samples (C_T values ≤ 38) with the antibiotics were inoculated into 9-10-day-old specific pathogen-free (SPF) embryonated chicken eggs (Beijing Merial Vital Laboratory Animal Technology Co., Ltd., Beijing, China) for virus isolation. Allantoic fluid were harvested after 3 days, and influenza virus were detected by hemagglutination assays with turkey erythrocytes.

Sequencing analysis and subtype determination. Cultured influenza viruses were analyzed further by a one-step conventional RT-PCR using universal primers targeting each of the eight influenza segments[34], using a one-step RT-PCR Kit (Cat. No. 055A, TaKaRa). Amplified RT-PCR products were purified using a QIAquick Gel Extraction Kit (Cat. No. 28704, Qiagen), subcloned into a pGEM®-T Vector System I (Cat. No. A3600, Promega) and sequenced using standard techniques on an Applied Biosystems ABI 3730 DNA genetic analyzer. Sequences were then assembled and edited using Lasergene version 7.0 (DNASTAR, Madison, WI, USA), these sequences were then compared with similar virus sequence from GenBank to determine viral subtype.

Molecular clock and temporal phylogenetic analysis. To infer the evolutionary histories for each gene segment (PB2, PB1, PA, HA, NP, NA, MP, and NS), we used BLAST[35,36] with the default parameters to find the 200 most similar viral sequences to the novel H2N2 virus. These sequences were downloaded from NCBI[24] and GISAID (http://gisaid.org), then aligned with Muscle v3.8.31[37] followed with manual alignment to codon position in the coding regions. For segments NS and MP, the aligned sequences were edited such that all of the codons in first open reading frame (ORF) were followed by the remaining codons in the second ORF. Genetic distances were calculated using the program "dnadist" and phylogenies were estimated using the program "neighbor" with the default parameters in Phylip 3.68 (version 3.2), with rooting chosen to maximize the regression fit between the isolation date of each influenza sequence and the genetic distance from that sequence to the root of the phylogeny[38,39]. Residual analysis was performed to identify and remove significant outliers, which most likely result from isolation data annotation errors in the sequence database. The high correlation between the isolation date of each influenza sequence and its genetic distance to the root suggest a "molecular clock" for the closely related viral sequences. The temporal phylogenies and rates of evolution were inferred using the software BEAST v1.75[40]. BEAST was used to sample phylogenies and the dates of divergences between viruses from their joint posterior distribution, in which the sequences are constrained by their known date of sampling. The codon-position-specific HKY and Gamma +4 nucleotide substitution model and the uncorrelated lognormal molecular clock model were used in the analysis. The results were similar when both the SRD06 and Yang96 substitution models were used. For the analyses using Bayesian MCMC sampling, in all cases chain lengths were run for sufficient time to achieve convergence both by manual checking and by the effective sample size method, which was recommended to be greater than 200 in BEAST. Furthermore, at least two independent runs of each gene segment were performed and compared to ensure stable results.

Receptor binding assays. Receptor binding specificity was analyzed by a solid-phase direct binding assay biotinylated sialylglycopolymers: 3′-sialyllactose-PAA-biotin (3′SL-PAA, 3′ Neu5Acα2-3Galβ1-4Glc) and 6′-sialyllactosamine-PAA-biotin (6′SLN-PAA, 6′ Neu5Acα2-6Galβ1-4Glcβ) (Cat. No. 01-038, 01-039, Glycotech, Gaithersburg, MD) as previously described[27]. Briefly, serial dilutions (0.001875 μm/ml, 0.00375 μm/ml, 0.0075 μm/ml, 0.015 μm/ml, 0.03 μm/ml, 0.06 μm/ml, 0.12 μm/ml, and 0.24 μm/ml) of 3′-sialyllactose-PAA-biotin and 6′-sialyllactosamine-PAA-biotin were prepared in PBS; 100 μl of this dilution was added to each well of the 96-well microtiter plates (Cat. No. costar 30500, Thermo Fisher, MA, USA) and allowed to attach overnight at 4°C. After removal of the glycopolymer solution, the plates were blocked with 0.1 ml of PBS containing 2% bovine serum albumin (BSA) at room temperature for 1 hour. After washing with ice-cold PBS containing 0.1% Tween 20 (PBST) and cold PBS, the plates were incubated in a solution containing influenza virus (64 HA units in PBS containing 2% BSA) at 4°C overnight. After washing with cold PBST and cold PBS, chicken antisera against A/WX/2/2013 (H2N2), A/Changchun/XD/2009 (H1N1), and A/Chicken/Jilin/Hu-3/2006 (H9N2) viruses were added to each well and the plates were incubated at 4°C for 2 hours. The wells were washed with ice-cold PBST and cold PBS then incubated with HRP-linked goat-anti-chicken antibody (Sigma-Aldrich, www.sigmaaldrich.com) for 2 hours at 4°C. After washing with ice-cold PBST and cold PBS, the plates were incubated 200 μl of TMB substrate (Cat.No.T8665-100Ml, Sigma-Aldrich) for 20 minutes at room temperature. The reaction was then stopped with 100 μl of 50mM H_2SO_4 and the optical density was measured at 450 nm.

Pathogenicity of avian H2N2 virus in chickens. *In vivo* pathogenicity studies on the avian H2N2 isolate were performed in accordance with the World Organization for Animal Health (www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.04_AI.pdf). In brief, ten 6-week-old SPF chickens (Beijing Merial Vital Laboratory Animal Technology Co., Ltd., Beijing, China) were inoculated intravenously with 0.1ml 10^6 Egg 50% infective doses (EID_{50}) of amniotic allantoic fluid. Two chickens were inoculated with PBS to be used as negative controls. The chickens were observed daily for sickness or death for 10 days post inoculation (p.i), which is used to determine the intravenous pathogenicity index (IVPI). At each observation each chicken was scored 0 if normal, 1 if sick, 2 if severely sick, and 3 if dead. Chickens were determined to be sick if one of the following signs were observed, and severely sick of two or more of the signs were observed: respiratory distress, sneezing, coughing, diarrhea, and cyanosis of exposed skin or wattles, edema of the face or head, and nervous signs. Chickens found to be too sick to eat or drink were euthanized humanely and scored dead at the next observation. The IVPI was calculated as the mean score per chicken per observation over the 10-day period. Viruses with an IVPI greater than 1.2 were considered to be highly pathogenic. The serum sample of each chicken was collected to determine the HI titer of the novel H2N2 virus on day 10 p.i.

Pathogenicity of avian H2N2 virus in mice. Six- to seven-week old SPF BALB/c mice (Vital River Laboratories Technology Co., Ltd., Beijing, China) were used for infectivity experiments. Animals were weighed and anesthetized with diethyl ether before intranasal inoculation with 50 μl of 10^1-$10^6 EID_{50}$ of the novel H2N2 viruses. A total of 66 BALB/c mice were divided into 6 groups (11 mice for each group) and were inoculated intranasally with the novel H2N2 virus, with doses ranging from 10^6 EID_{50} to 10^1 EID_{50}. The weight of each mouse was recorded once daily, and the general health status of each mouse was observed twice daily. After onset of disease, the general health status of each mouse was observed three times per day. Three mice of each group were killed on day 3 p.i and three killed on day 5 p.i. At necropsy tissue specimens from the brain, lung, kidney, and spleen were cultured for virus and fixed

for histological study. Surviving mice were euthanized on day 14 p.i, and the lungs and serum samples preserved at −80°C.

Transmission of H2N2 virus in guinea pigs. Infection and transmissibility of the novel H2N2 virus was tested in twelve guinea pigs, which were virus antibody free (Vital River Laboratories Technology Co., Ltd., Beijing, China). Blood samples from each guinea pig were collected before intranasal inoculation of the novel H2N2 virus, with doses ranging from 10^1 to 10^6 EID_{50}. For the contact transmission study, 3 guinea pigs infected with 300 μl of 10^6 EID_{50} of H2N2 virus and 3 naïve guinea pigs were placed in the same cage at 24 hours p.i. Nasal, oral, and rectal swabs were collected and body temperature measured from each animal on days 2, 4, 6, 8, 10, and 12 p.i. The aerosol transmission study used the same protocol as the contact transmission study, except the 3 infected guinea pigs were placed in an adjacent cage to the 3 naïve guinea pigs (5 cm away). Swabs and tissue cultures were inoculated into SPF embryonated eggs. EID_{50} values were calculated by the Reed-Muench method[41]. A serum sample from each guinea pig was collected on day 14 p.i.

Hemagglutination inhibition (HI) Assay. HI assays were performed on sera from live poultry and humans in Wuxi City, to determine the prevalence of previous H2N2 influenza infection. The sera samples were pre-treated with receptor-destroying enzyme RDE (II) "Seiken", (Cat. No. 370013, Denka Seiken Co. Ltd., Tokyo, Japan) at 37°C for 18 hours and then heat-inactivated at 56°C for 30 minutes to eliminate nonspecific inhibitors. The sera samples were next adsorbed with 1% horse red blood cells. Similarly, the sera of mice and guinea pigs in the pathogenicity and transmission studies were examined for evidence of H2N2 virus infection. A HI titer ≥ 1:40 was considered as positive.

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Author contributions

M.J.M., J.Q., and W.C.C. conceived and designed the experiments. M.J.M., X.X.Y., Y.H.Q., S.Y.Z., S.H., T.C.W., S.H.C., G.Y.M., X.Y.S., L.N.L., A.P.W., T.J.J., T.Z., J.L.W., X. L., and Y.W.G. performed the experiments and analysed the data. M.J.M., S.H., and W.C.C. wrote the manuscript. B.L., Y.H.Q., A.P.W., T.J.J., and G.C.G. edited the manuscript.

Additional information

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