SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
MOLECULAR EVOLUTION
GENE AMPLIFICATION

Received
22 August 2013

Accepted
28 November 2013

Published
17 December 2013

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Phylogenetic and molecular characterization of chicken anemia virus in southern China from 2011 to 2012

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Chicken anemia virus (CAV) is an important pathogen that causes severe immunosuppression in young chickens. We have characterized 13 CAVs isolated from different commercial farms in southern China between 2011 and 2012. We discovered 92 variable residues compared to 37 other CAV complete genome sequences from other parts of the world listed in GenBank; these residues have not been previously observed. All of the Chinese CAV genomes that were characterized in this study had a glutamine at position 394, a hallmark of highly pathogenic CAVs. We also discovered that intra-group genetic recombination plays a role in generating genetic diversity in natural populations of CAV. The GD-J-12 isolate was a possible recombinant between GD-C-12 and GD-M-12 in the genomic region that encompassed both the coding and non-coding regions.

Chicken anemia virus (CAV) is a transmissible agent that produces severe anemia with aplasia of the bone marrow and atrophy of the lymphoid organs in susceptible chickens[1]. CAV causes severe anemia in newly hatched chickens and destruction of erythroblastoid cells in the bone marrow and thymocytes in the thymic cortex[2]. Some broiler flocks examined at the time of slaughter had antibodies against CAV, suggesting vertical and horizontal modes of transmission[3,4]. CAV isolates were thought to belong to a single serotype and were antigenically indistinguishable by serum neutralization tests[5]. In 2011, a new human virus, named human gyrovirus (HGyV) due to its homology with CAV, was identified on the surface of human skin[6]. CAV (JQ690762) was identified in pediatric fecal samples in Beijing, China, in 2012[7], and the genome had a 21-nt insertion (TCCGTACAGGGGGGTACGTCA) in comparison with the strain named GD-1-12 (accession no. JX260426), which was isolated in Southern China in 2012[8]. As of 2012 in China, two gyrovirus species have been designated GyV3 and GyV4. GyV3 was detected in samples of diarrhea and normal feces from Chilean children in the USA, and these had a low level of sequence similarity with other gyroviruses, which may reflect the consumption of CAV-infected/vaccinated chickens[9]; GyV4 was identified in human stool and in chicken meat sold for human consumption in Hong Kong in 2012[10]. In the same year, HGyV DNA was detected in four samples in Italy[11]. These data reflect the potential for CAV to threaten human health, especially after the consumption of infected or CAV-vaccinated chickens. CAV is distributed worldwide with variable prevalence, causing significant economic losses in the production of young birds. In China, CAV was first isolated in 1996[12]. Ducatez confirmed a high prevalence of approximately 87% in live bird markets in Southeast China[13]. However, there are no reports to date on either the molecular characteristics or the complete genomic characterization of CAV in southern China.

CAV has been classified under the genus *Gyrovirus* and the family *Circoviridae*[14]. CAV is a non-enveloped, icosahedral virus approximately 25 nm in diameter, with a negative-sense, single-stranded circular DNA genome approximately 2298 to 2319 nucleotides in length[15]. The genome consists of three major partially overlapping, open reading frames that encode for peptides of 51.6 (VP1), 24 (VP2) and 13.6 (VP3) kDa[16]. VP1 is the major viral structural protein, VP2 is a scaffolding protein and VP3 is a non-structural protein named apoptin. VP1 and VP2 are the protective proteins that induce neutralizing antibodies[17]. The VP1 gene has the highest variability of the three overlapping ORFs, according to sequences that have been submitted to GenBank[18]. Based on phylogenetic analyses of the entire coding regions of VP1, VP2 and VP3, the sequences were organized into four distinct groups (A–D) and five subgroups (A1, A2, A3, D1 and D2)[19]. Studies have confirmed that a hypervariable region spanning 13 amino acids in VP1 from position 139 to 151 affects viral growth and spread, especially residues

139 and 144. These residues are believed to play major roles in this regard considering that VP1 Q139 and/or Q144 show a decreased capacity to spread[20].

Genetic diversity in *Gyrovirus* is due to adaptive evolution driven by high mutation rates and genetic recombination. Larger scale sequence changes can occur via the exchange of genetic information with other related viruses via the process of recombination[21,22]. Compared to other DNA viruses, *Circoviridae* appears to have relatively low rates of recombination. To date, only two studies have reported recombination in CAV. However, one was performed in 2007[23], while the other focused on the open reading frame structure and inter-subtype recombination analysis[24]. In the present study, we analyzed the complete genome sequences of CAV from different geographical locations in GenBank in order to gain insights into the presence of recombination among CAV strains. The genetic and recombination characterization of CAVs from different geographical locations is pivotal to our understanding of CAV evolution.

Results

Virus detection and isolation. Thirteen viruses were identified as CAV from fifteen thymus and eight spleen samples by PCR analyses. The 13 CAVs were submitted to virus isolation in vitro in MDCC-MSB1 cells. After cells were cultured 3 days, we harvested the viruses, and their presence was confirmed by PCR analysis with the NC1 and NC2 primers. Accordingly, all thirteen viruses were confirmed to be present. Among the 13 CAVs, the nine strains (GD-C-12,GD-D-12, GD-E-12,GD-F-12,GD-H-12,GD-J-12,GD-L-12,GD-M-12,GD-N-12) were isolated in Guangdong province, China, 2012. The GD-B-12 and GD-I-12 strains were isolated in Guangxi province, China, 2011. The GD-G-12 strain was isolated in Hainan province, China, 2011 and the GD-K-12 strain was isolated in Hainan province, China, 2012.

PCR amplification of the complete genome of CAVs. PCR amplification of the complete genome of 13 positive CAV isolates using primers CQ1F and CQ1R yielded a specific 1,778 bp product, and the CQ2F and CQ2R primers yielded a specific 831 bp product as expected.

Sequence accession numbers. The CAV genome sequences characterized in this study were submitted to GenBank with the following accession numbers: KF224926, KF224927, KF224928, KF224929, KF224930, KF224931, KF224932, KF224933, KF224934, KF224935, KF224936, KF224937 and KF224938.

Complete sequence alignment and phylogenetic analysis. The nucleotide sequence identities between the genomes of the 13 CAV isolates and the genomes of 37 reported CAV isolates from GenBank ranged from 95.7%–99.7%. The minimum diversity was 0.3% between the GD-1-12 and GD-G-12 isolates, and the maximum diversity was 4.4% between GD-K-12 and three isolates (GD-L-12, GD-M-12 and GD-N-12). We identified 92 nucleotide mutations compared to the 37 other complete CAV sequences; these mutations had not been observed previously: nt positions 14, 80, 105, 106, 116, 119, 122, 123, 124, 164, 169, 181, 182, 187, 195, 251, 267, 287, 308, 318, 361, 367, 400, 416, 417, 500, 504, 513, 518, 531, 535, 536, 537, 538, 542, 544, 563, 644, 659, 696, 747, 863, 864, 941, 969, 994, 1012, 1053, 1056, 1059, 1066, 1137, 1245, 1257, 1269, 1270, 1298, 1323, 1330, 1393, 1395, 1396, 1434, 1452, 1457, 1485, 1493, 1610, 1740, 1774, 1797, 1810, 1839, 1840, 1847, 1905, 1923, 1951, 2013, 2016, 2085, 2173, 2185, 2191, 2194, 2201, 2202, 2204, 2214, 2259, 2275, 2278. Common polymorphisms that we observed included the following: T14C in isolates GD-D-12 and GD-F-12; nine isolates (GD-B-12, GD-C-12, GD-D-12, GD-E-12, GD-F-12, GD-H-12, GD-I-12, GD-K-12, GD-L-12) contained the C122G polymorphism; C123G was confirmed in the GD-D-12, GD-E-12 and GD-I-12 isolates. T181C was confirmed in isolates GD-H-12 and GD-M-12; A187G in isolates GD-C-12 and GD-E-12; G251A in isolates GD-H-12 and GD-I-12; T308A in isolates GD-H-12 and GD-I-12; G941A in isolates GD-L-12 and GD-N-12; C1137A in isolates GD-H-12 and GD-I-12; and C1270T in isolates GD-D-12 and GD-I-12; C1434T in isolates GD-H-12 and GD-I-12; T1740C in isolates GD-H-12 and GD-I-12 isolates. Three isolates (GD-E-12, GD-G-12 and GD-H-12) contained the C2204A polymorphism.

Among the 13 CAV isolates, we identified 187 nucleotide mutations. We also found that there were 83 common nucleotide mutations between the 187 mutations among the 13 CAV sequences and 92 mutations compared to 37 other CAV complete genome sequences. They were T14C, T80C, C105T, G106C, C116A/G, T119G/A, G122C, C123G, C124G, C164T, C169T, T181C, C182A, A187G, T195C, G251A, A267G, T308A, A318G, G361A, G367C, G416A, A417G, A500G, A504G, A513T, A518C, C531G, C535A, G536C, A537T, T538G, A542T, C544T, A644G, A659C, A696G, G941A, G969A, G994C, C1012T, A1053C, C1056A, C1059T/A, C1137A, T1245C, C1257T/A, G1269T, C1270T, A1298G, A1323G, A1330G, A1393C, G1395A, A1396G, C1434T, A1452G, A1457G, G1485A, T1493C, G1610A, T1740C, A1774G, A1797G, C1839G/T, T1840C, A1847G, G1905C, C1923A, T1951G, G2013A, C2016T, C2173A, G2185A, G2191T, G2194T, C2201G/A, C2202G, A2204C, A2214C, A2259T, A2275T and A2278G. There were nine different nucleotide mutations between the 92 positions compared to 37 CAV sequences and the 83 common mutations. They were C287G, A400G, C563T, T747C, G863A, A864T, A1066G, C1810G and C2085G. However, 187 nucleotide mutations were identified among the 13 CAV isolates at position 566; isolates GD-C-12, GD-D-12, GD-E-12, GD-F-12, GD-G-12, GD-H-12, GD-I-12, GD-J-12, GD-K-12, GD-L-12, GD-M-12 and GD-N-12 all showed C566G.

The complete phylogenetic tree was obtained by aligning the 37 sequences, and it resulted in two major groups (Fig. 1a): one with eleven strains (group II; AY839944, DQ141670, JQ690762, DQ124935, AF395114, AF311900, DQ124936, DQ217400, AF285882, CAU65414, AB02740) and another with the remaining 25 sequences (group I). Interestingly, with the exception of GD-K-12, all of the 12 southern Chinese isolates were clustered in the large cluster that showed intra-group recombination.

Nucleotide alignment of different coding regions of the CAV genome. The complete nucleotide sequences of VP1, VP2 and VP3 were obtained from the full-length genes based on the sequence information in GenBank. The complete sequences of the VP1, VP2 and VP3 genes were 1350, 651 and 366 nucleotides long, respectively, and contained no insertions or deletions.

The VP1 sequences of the southern Chinese CAV isolates contained 40 nucleotide mutations that had not been previously observed; these were located at residues 32, 33, 110, 138, 163, 181, 222, 225, 228, 235, 306, 414, 426, 438, 439, 467, 492, 562, 564, 565, 621, 626, 654, 662, 779, 909, 943, 966, 979, 1008, 1009, 1016, 1071, 1074, 1092, 1167, 1182, 1185, 1254 and 1342. C228/306A was found in two CAV isolates from southern China (GD-I-12 and GD-H-12), while GD-J-12 and GD-K-12 had different mutations at position 426. GD-H-12 and GD-I-12 both had the mutation T909C. The VP2 region contained 25 variable positions that were detected in the 13 isolates from southern China, at positions 3, 42, 58, 136, 142, 146, 155, 160, 173, 177, 178, 179, 180, 184, 186, 205, 208, 286, 301, 338, 389, 506, 583, 611 and 636. Analysis of the 366 nucleotides of VP3 revealed 18 variable positions, which were specific to the southern China CAV sequences (nts 30, 36, 40, 49, 54, 67, 71, 72, 73, 74, 78, 80, 99, 102, 180, 195, 232 and 283).

Amino acid alignment of different coding regions of CAV genome. When the amino acid sequences of the 13 CAV genes were compared with those of representative CAV strains, it was clear that the 13 isolate strains all shared 52 amino acid variations (at positions 5, 27, 36, 39, 40, 41, 42, 55, 61, 63,84, 88, 96, 103, 134,

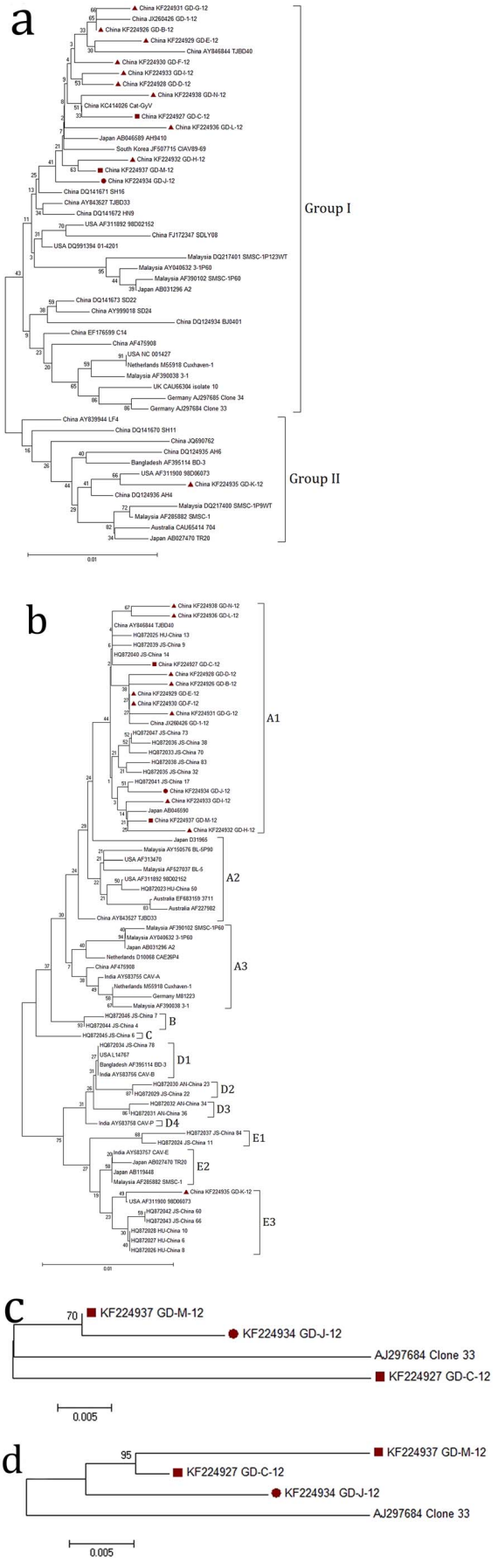

Figure 1 | (a) Phylogenetic analysis of the complete sequences of 37 CAV isolates from different parts of the world listed in GenBank. The ten southern Chinese isolates (GD-B-12, GD-D-12, GD-E-12, GD-F-12, GD-G-12, GD-H-12, GD-I-12, GD-K-12, GD-L-12, GD-N-12) are marked by red triangles. The isolate GD-J-12 is marked by a red circle. Two isolates (GD-C-12 and GD-M-12) are represented by red squares. (b) Phylogenetic analysis of the VP2, VP3 and partial VP1 genes of 54 CAV strains from different parts of the world listed in GenBank. The ten southern Chinese isolates (GD-B-12, GD-D-12, GD-E-12, GD-F-12, GD-G-12, GD-H-12, GD-I-12, GD-K-12, GD-L-12 and GD-N-12) are marked by red triangles. The isolate GD-J-12 is marked by a red circle. Two isolates (GD-C-12 and GD-M-12) are represented by red squares; (c and d) represent the recombinant region (2108–44) and the non-recombinant region (45–2107), respectively. The putative recombinant is shown with circles, and the putative parental lineages are shown with squares. The Cux-1 strain was used as an outgroup. The whole sequence was analyzed using MEGA 5.1 software with neighbor-joining (NJ) phylogenetic tree methods together with the sequences characterized in this study. Each tree was produced using a consensus of 1000 bootstrap replicates.

139, 165, 167, 177, 179, 180, 181, 182, 188, 189, 215, 220, 290, 314, 332, 356, 423, 424, 433, 444, 465, 466, 486, 498, 537, 592, 604, 616, 651, 724, 728, 730, 731, 733, 734, 747 and 760). At position 40, the GD-F-12 and GD-M-12 strains had amino acids G and E, respectively. Amino acid variation based on the VP1 sequence (450 AA) indicated an overall variation of 0.7–4.0% among all isolates compared with the 37 CAV sequences. In the hypervariable region of VP1 from position 139 to 151, the GD-D-12 isolate showed two mutations, W146C and P147S, while the GD-I-12 isolate only contained a single mutation in the hypervariable region (P147S). Residue 394 in VP1 was a major genetic determinant of virulence, with glutamine (Q) and histidine (H) representative of highly pathogenic and less pathogenic viruses, respectively[25,26]. All of the southern Chinese CAV isolates contained a glutamine at position 394, suggesting that they all represented highly pathogenic viruses. The amino acids at positions 139 and 144 play vital roles in virus growth and spread, considering that VP1 Q139 and/or Q144 are associated with decreased spread[20]. Only the GD-K-12 isolate had both Q139 and Q144, which indicated that the GD-K-12 isolate had low rates of growth and spread. However, the other 12 CAVs (GD-B-12, GD-C-12, GD-D-12, GD-E-12, GD-F-12, GD-G-12, GD-H-12, GD-I-12, GD-J-12, GD-L-12, GD-N-12 and GD-M-12) all contained the residues K139 and E144. Compared with VP1 sequences, VP2 and VP3 were relatively conserved. The VP2 and VP3 sequences of the GD-G-12 strain shared 99.5% and 99.2% maximum amino acid identify to GD-H-12, respectively.

Nucleotide sequence alignment and phylogenetic analysis of the VP2, VP3 and partial VP1 genes. In addition to a complete sequence alignment and phylogenetic analysis of the 13 CAV isolates, we also performed sequence alignment and phylogenetic analyses of the VP2, VP3 and partial VP1 genes compared to the 54 other complete CAV sequences listed in GenBank. The nucleotides of the 54 other complete CAV genomes included 1,766 bp that contained almost the entire CAV coding region with the exception of a few amino acids at the C terminus of VP1. There were 54 nucleotide mutations compared to the 54 other complete CAV sequences; these mutations had not been observed previously: nt positions 3, 42, 58, 59, 142, 146, 155, 160, 173, 177, 178, 179, 180, 184, 186, 205, 208, 286, 301, 338, 389, 506, 583, 611, 636, 654, 698, 708, 779, 887, 899, 911, 912, 940, 965, 972, 1035, 1037, 1038, 1099, 1127, 1135, 1252, 1382, 1400, 1416, 1439, 1452, 1482, 1489, 1495, 1514, 1543 and 1544. We also identified 136 nucleotide mutations in the 13 CAV isolates. There were 51 common nucleotide mutations in the 13 CAV isolates compared to the previously identified 54 nucleotide mutations in the 54 other complete CAV sequences. They were G3A, A42G, G58A, A59G, A142G, A146G, A155T, A160C, C173G, C177A, G178C, A179T, T180G, A184T, C186T,

C205T, A286G, A301C, A338G, T389C, A506T, G583A, G611A, G636C, C654T, C698A, A708G, C779A, T887C, C899T, G911T, C912T, A940G, A965G, A972G, A1035C, G1037A, A1038G, G1099A, G1127A, T1135C, G1252A, T1382C, A1400G, C1452G, T1482C, A1489G, A1495G, C1514T, T1543C and G/T1544A.

The phylogenetic analysis of the 13 CAV genomes characterized in this study, together with the 54 CAV strains from different countries, led to the establishment of five distinct sequence groups (A, B, C, D and E), with bootstrap values of 37, 93 and 75. Group A could be further divided into subgroups A1, A2 and A3, group D was divided into subgroups D1, D2, D3 and D4, and group E was divided into subgroups E1, E2 and E3. The 13 CAV sequences were located as two different clusters in groups A1 and E3. Except for GD-K-12, all of the 12 southern Chinese isolates were clustered in group A1, which meant that group A1 was the main southern Chinese branch (Fig. 1b).

Group A represented a major group of 42 isolates that originated from China, Japan, Malaysia, the USA, Australia, India, Germany and the Netherlands. Group B represented two isolates that originated from China. Group C represented a single isolate which originated from China. Group D represented nine isolates that originated from China, the USA, Bangladesh and India. Group E represented 13 isolates that originated from China, Japan, India, Malaysia and the USA.

Putative recombination breakpoint detection in intra-group. In order to identify recombination within the genomes, an initial phylogenetic tree was constructed based on the 50 CAV sequences from around the world. Phylogenetic analyses suggested that GD-C-12, GD-J-12 and GD-M-12 clustered in group I (Fig. 1a). Recombination might have occurred in the partial coding region and the partial non-coding region (Fig. 2a and 2b). As illustrated in Fig. 2, recombination between the lineages represented by the isolate GD-C-12 of group I as the major parent and GD-M-12 of group I as the minor parent, led to the recombinant isolate GD-J-12 with the recombination breakpoints mapping to positions 2,108 (beginning breakpoint) and 44 (ending breakpoint). To gain insight into possible recombination events, phylogenetic profile analysis of the recombinant viruses was carried out using the Simplot program (Fig. 2c). To confirm the results of the Simplot analysis, we extracted positions on opposite sides of the breakpoint from the alignment, that is, positions 2108–44 and 45–2107, respectively, to determine the statistical likelihood of recombination. The AJ297684 strain was used as an outgroup. GD-J-12 clustered together with GD-M-12 only when the recombinant region was considered for tree construction, whereas in a tree representing only the non-recombinant region, GD-C-12 clustered with GD-M-12 (Fig. 1c and 1d). The differences in the phylogenetic relationships point towards a possible recombination event in GD-J-12. According to the gene structure of CAV (Fig. 3), the recombinant region spanned the partial VP1 region and partial non-coding region. Studies have confirmed that CAV recombination is only found inter-group[23,24], but our research identified an intra-group recombination event among CAVs, which has not been previously described.

Discussion

The first report of CAV was from contaminated vaccines in Japan[27]. In China, CAV was first isolated in 1996[12]. CAV is an infectious agent that affects commercial poultry and has a worldwide distribution. Argentina[28], South Korea[29], India[14], Nigeria[15] and other countries have reported outbreaks in commercial flocks. The complete genomes of 13 CAVs from southern China were sequenced. Analysis of the CAV genes revealed that, with the exception of the GD-K-12 isolate, the other 12 CAVs all belonged to a single phylogenetic group (group I), which indicated that these isolates were the dominant prevalent strains. In our study, we also compared the 13 CAV sequences to the 54 other complete CAV sequences that are listed in GenBank which contain most of the VP1, VP2 and VP3 gene sequences. Genetic typing according to partial CAV sequences has been used as a means of tracing the spread of CAV[14,26]. In China, previous research only focused on either the VP1 gene or the approximately full-length CAV genomes. In 2012, we analyzed the complete genome sequence of one strain isolated in southern China[8]. Analysis of the complete genomes of 13 southern Chinese CAVs produced a total of 92 unique mutations, which had not been previously observed, compared to 37 other CAV sequences from GenBank. Analysis of the 450 amino acids of the VP1 region produced 18 amino acid variations that were specific to the viral genomes of CAVs from southern China. Only three of the variations observed in two sequences (GD-D-12 and GD-I-12) were in the hypervariable region. However, the genome sequences of VP2 and VP3 were relatively conserved compared with VP1. A study[30] that determined the complete nucleotide sequence of a CAV strain concluded that the N-terminal half of VP3 and the N-terminal three-quarters of VP2 are well conserved and might sustain an essential function of these proteins. It also suggested that amino acid changes in VP1 might influence the antigenic variations among CAV isolates. The amino acid at position 394 in VP1 was reported[31] to be a major genetic determinant of virulence. The 13 southern Chinese strains in our study had a Q at position 394. The complete genomes of 13 isolates from southern China shared nucleotide variations ranging from 0.3% to 4.3% with the 37 relevant sequences from GenBank.

Recombination is considered to be a major source of variation and the driving force for the evolution of several viral families. As a DNA virus and member of the family *Circoviridae*, CAV has rarely been found to show recombination. Genetic analysis of the CAV strains isolated in China by Eltahir[24] was limited to the complete VP1, VP2 and VP3 coding regions; it confirmed that recombination had occurred in the coding regions of CAV, but they did not analyze the complete genome of CAV, which contains the non-coding region. Eltahir and colleagues[23] performed a phylogenetic analysis of full-length CAV strains isolated to detect possible recombination events, and their analysis demonstrated that a recombination event occurred in the coding region. Our results provided strong evidence that not only does recombination occur in the coding region, but that it also occurs in the non-coding region. Most importantly, we provide evidence of natural intra-group genetic recombination in CAV, which further suggests that the coding and non-coding regions examined are subjected to undergoing genetic recombination of the complete CAV genome. Furthermore, the recombinant gene must have undergone virus selection and have prevailed over parental strains, suggesting greater levels of fitness and epidemic spreading[36]. Recombination may be one of the important mechanisms in generating genetic variation in CAV and recombination has important implications for detection and vaccination strategies. Given the high prevalence rate of CAV infection, further analyzing the pathogenicity of the recombinant virus will be conducted in future studies.

Taken together, we have found evidence that recombination occurs among CAV isolates across their entire genome in geographically diverse poultry flocks. It is important to have recent data on the extent of variability of CAV in southern China, as it can help in monitoring the spread of the virus and in making decisions regarding treatment strategies in humans.

Methods

Clinical samples. From 2011 to 2012, fifteen thymus and eight spleen samples were collected from diseased young and adult chickens. Samples were isolated from different farms in three provinces (Guangdong, Guangxi and Hainan) of southern China. The diseased chickens showed signs of atrophy of the thymus, pale bone marrow, and a swollen and discolored liver at necropsy. Samples were collected and stored at −80°C.

DNA isolation and detection. Virus DNA was extracted from 23 suspected CAV isolates by using a commercial DNA extraction kit (QIAamp DNA Stool Mini Kit,

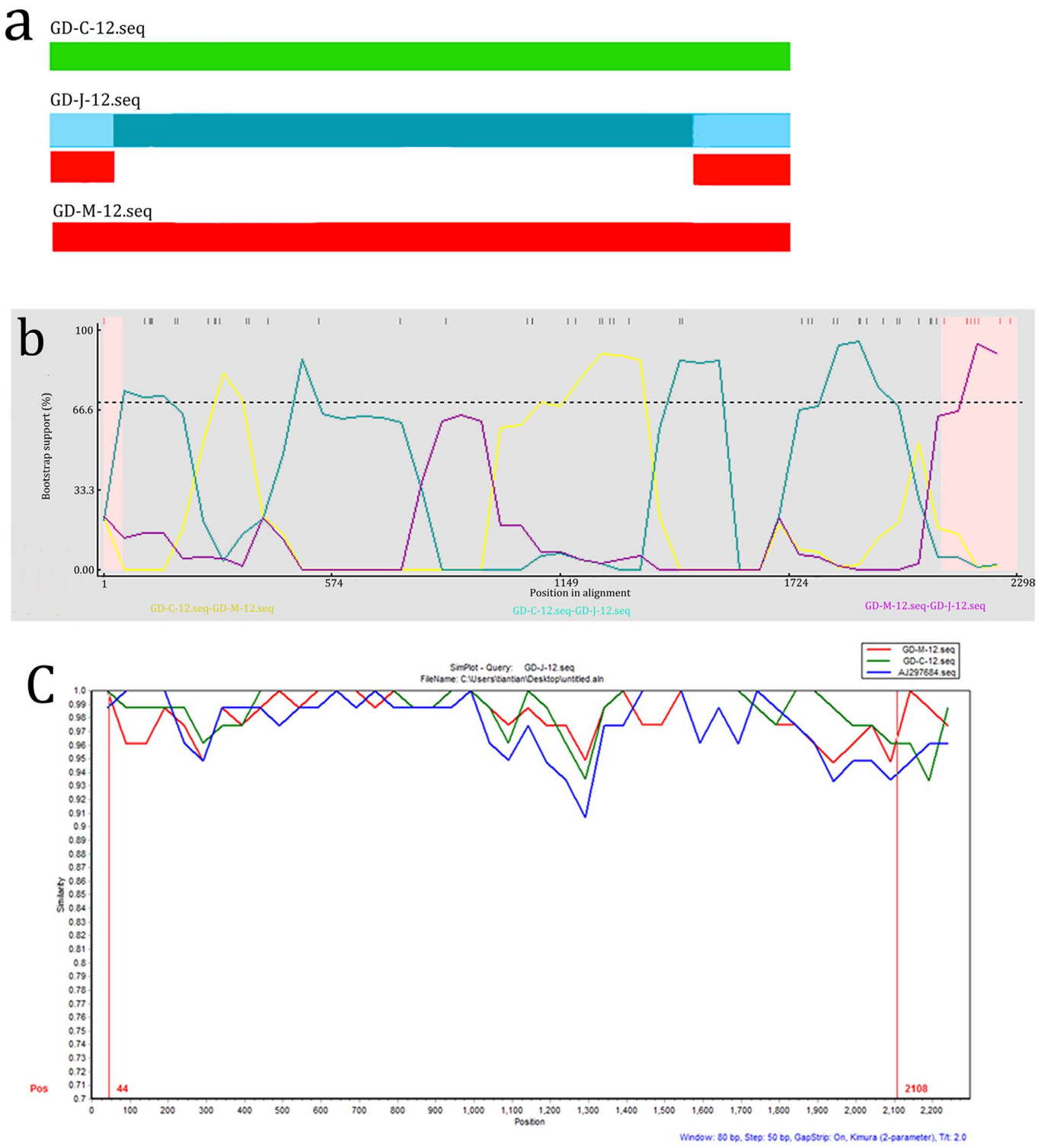

Figure 2 | (a and b) Bootscan analysis of the recombinant, and major and minor parent sequences. Bootscan was based on the pairwise distance model with a window size of 200, step size of 50 and 1000 bootstrap replicates generated by the RDP4 program. (c) A comparison of the three CAV isolates: GD-J-12, GD-C-12, GD-M-12. The GD-J-12 isolate was used as the query. The AJ297684 sequence was included as an outgroup. The y-axis gives the percentage of identity within a sliding window 80-bp wide centered on the position plotted, with a stepsize between plots of 50 bp.

QIAgen, Hilden, Germany) according to the manufacturer's instructions. The virus DNA was then quantitated and stored at −20°C until PCR was performed.

The extracted DNA was screened by PCR. The NC1 primer was 5′ CCTCTCTG GCAAAGTCCAAG 3′ and NC2 was 5′ CATCTGCCCATTTGATGTTG 3′. Primers were designed by Primer Premier 5.0 to amplify 735 bp from the partial coding regions of CAV. The PCR amplification was performed in a total volume of 25 μL, including 12.5 μL of buffer I, 4 μL dNTPs, 0.5 μL of each primer, 6 μL distilled water, 1 μL DNA and 0.5 μL LA Taq polymerase (TaKaRa, Biotechnology, Dalian, China). Amplification reactions were performed using the automated thermal cycler (Gene Amp PCR System 9700, Applied Biosystems, Foster City, CA, USA) with the following cycling profile: initial denaturation at 94°C for 5 min followed by 28 cycles of denaturation, annealing and extension at 94°C for 30 s, 56°C for 30 s and 72°C for 1 min, respectively, and a final extension step was performed at 72°C for 7 min. The 735-bp reaction product was analyzed on 1% agarose gels. Negative controls were included in every three PCR reactions and one positive control was included in each set of reactions.

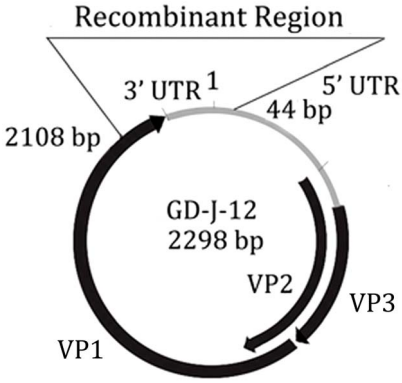

Figure 3 | Map of the GD-J-12 isolate and the recombination breakpoints (beginning breakpoint at 2108 and ending breakpoint at 44) in the alignment of the GD-J-12 DNA nucleotide sequence.

Virus isolation. Thirteen positive isolates were propagated in Marek's disease virus transformed MSB-1 cell line as previously described[32]. First, 0.5 ml of virus was mixed with the MSB-1 cell pellet resuspended in 0.5 ml of 1640 medium and incubated at 37°C for 1 h. Then, 5 ml of 1640 medium was added to the mixture, and the cells were suspended and incubated at 37°C for 3 days. Then, a 1 ml cell suspension was transferred to 4 ml of fresh 1640 medium and incubated. Cells were passaged repeatedly to measure the stable cytopathic effect and a high virus titer was obtained.

Amplification, cloning and sequencing of the positive CAV genomes. The viral DNA was further analyzed by two pairs of primers to amplify the complete nucleotide sequences of CAVs. Primers CQ1F 5′-CAATCACTCTATCGCTGTGT-3′ and CQ1R 5′-TTCGTCCATCTTGACTTTCT-3′ and CQ2F 5′-GGCTACTATTCCATC(A/T)CCATTCT-3′ and CQ2R 5′-GCTCGTCTTGCCATCTTACA-3′ were designed to amplify 1778-bp and 831-bp fragments, respectively, covering the entire nucleotide sequence. The PCR amplification was carried out in a 50 μl volume containing 25 μl buffer I, 16 μl dNTPs, 0.5 μl of each primer, 13.5 μl distilled water, 1 μl of the DNA and 0.5 μl LA Taq polymerase (TaKaRa, Biotechnology, Dalian, China). Amplification of the 831-bp region was carried out with an initial denaturation of 94°C for 4 min followed by 30 cycles of denaturation, annealing and extension at 94°C for 30 s, 59°C for 30 s and 72°C for 2 min, respectively, and the final extension was carried out at 72°C for 10 min. The reaction to amplify the 1,778-bp region contained 35 cycles. The initial denaturation was carried out at 94°C for 5 min. The cycle steps followed were 30 s at 94°C, 30 s at 58°C and 2 min 30 s at 72°C, and the final extension was carried out at 72°C for 10 min. All PCR amplification products were analyzed in 1% agarose gels stained with ethidium bromide. All reactions were performed at least in duplicate. PCR products were purified using the Gel Band Purification Kit (Omega Bio-Tek, American) and cloned into the pMD19-T vector (TaKaRa Bio Inc., Japan) followed by sequencing in triplicate using an ABI 3730 Sanger-based genetic analyzer (Carlsbad, CA, USA).

Sequence alignment. The DNA sequences and amino acid sequences (VP1, VP2 and VP3) were assembled using DNAStar (version 7; Madison, WI, USA), and a multiple-sequence alignment was performed with the Clustal W (BioEdit version 7) program. A neighbor joining (NJ) tree based on the full-length nucleic acid was constructed using the MEGA 5.1 program[33]. The robustness of the NJ tree was evaluated by bootstrap analysis of 1000 replicates.

Analysis of genetic recombination. We used the Recombination Detection Program (RDP4) v.4.1.3[34] for the detection of potential recombinant sequences, identification of candidate parental sequences and localization of possible recombination break points. Five different methods (RDP, GENECONV, MaxChi, Bootscan, and 3Seq) with a very high degree of confidence were used to detect the recombination event of the southern Chinese isolates. All six methods implemented in RDP4 were executed with default parameters, except that the multiple-comparison-corrected *P*-value cut-off was set to 0.01. In addition, Simplot version 3.5.1 was used to further confirm the results[35] and examine the southern Chinese CAV genomes for evidence of networked relationships using the MEGA 5.1 program.

Ethics statement. Institutional and national guidelines for the use and care of laboratory animals were closely followed. The use of animals in this study was approved by the South China Agricultural University Committee for Animal Experiments (approval ID: 201004152).

1. Taniguchi, T., Yuasa, N., Maeda, M. & Horiuchi, T. Chronological observations on hemato-pathological changes in chicks inoculated with chicken anemia agent. *Natl Inst Anim Health Q(Tokyo).* **23**, 1–12 (1983).
2. Kato, A., Fujino, M., Nakamura, T., Ishihama, A. & Otaki, Y. Gene organization of chicken anemia virus. *Virology.* **209**, 480–488 (1995).
3. Jørgensen, P. H., Otte, L., Nielsen, O. L. & Bisgaard, M. Influence of subclinical virus infections and other factors on broiler flock performance. *Br Poult Sci.* **36**, 455–463 (1995).
4. Todd, D. Circoviruses: immunosuppressive threats to avian species: a review. *Avian Pathol.* **29**, 373–394 (2000).
5. Yuasa, N. & Imai, K. Pathogenicity and antigenicity of eleven isolates of chicken anaemia agent (CAA). *Avian Pathol.* **15**, 639–645 (1986).
6. Sauvage, V. *et al.* Identification of the first human gyrovirus, a virus related to chicken anemia virus. *J Virol.* **85**, 7948–7950 (2011).
7. Yu, J. & Duan Z. Detection of chicken anemia virus and avian gyrovirus 2 in pediatric fecal samples. http://www.ncbi.nlm.nih.gov/nuccore/JQ690762.(20/5/2012).
8. Zhang, X. H. *et al.* Complete genome sequence analysis of a recent chicken anemia virus isolate and comparison with a chicken anemia virus isolate from human fecal samples in China. *J Virol.* **86**, 10896–10897 (2012).
9. Phan, T. G. *et al.* A third gyrovirus species in human faeces. *J Gen Virol.* **93**, 1356–1361 (2012).
10. Chu, D. K. *et al.* Characterization of a novel gyrovirus in human stool and chicken meat. *J lin Virol.* **55**, 209–213 (2012).
11. Maggi, F. *et al.* Human gyrovirus DNA in human blood, Italy. *Emerg Infect Dis.* **18**, 956–959 (2012).
12. Zhou, W., Yang, B., Shen, B., Han, S. & Zhou, J. A serologic survey of antibody against chicken infectious anemia virus by indirect immunofluorescent assay in domestic poultry in China. *Avian Dis.* **40**, 358–360 (1996).
13. Ducatez, M. F., Chen, H., Guan, Y. & Muller, C. P. Molecular epidemiology of chicken anemia virus (CAV) in southeastern Chinese live birds markets. *Avian Dis.* **52**, 68–73 (2008).
14. Natesan, S., Kataria, J. M., Dhama, K., Rahul, S. & Bhardwaj, N. Biological and molecular characterization of chicken anaemia virus isolates of Indian origin. *Virus Res.* **118**, 78–86 (2006).
15. Ducatez, M. F., Owoade, A. A., Abiola, J. O. & Muller, C. P. Molecular epidemiology of chicken anemia virus in Nigeria. *Arch Virol.* **151**, 97–111 (2005).
16. Noteborn, M. H. *et al.* Characterization of cloned chicken anemia virus DNA that contains all elements for the infectious replication cycle. *J Virol.* **65**, 3131–3139 (1991).
17. Koch, G., van Roozelaar, D. J., Verschueren, C. A., van der Eb, A. J. & Noteborn, M. H. Immunogenic and protective properties of chicken anaemia virus proteins expressed by baculovirus. *Vaccine.* **8**, 763–770 (1995).
18. Schat, K. A. Chicken infectious anemia. In: Saif, Y. M., Barnes, H. J., Glisson, J. R., Fadly, A. M., McDougald, L. R. & Swayne, D. E. (Eds.), *Disease of Poultry, 11th ed.* Iowa State University Press, Ames, Iowa, USA, pp. 182–202 (2003).
19. Eltahir, Y. M., Qian, K., Jin, W., Wang, P. & Qin, A. Molecular epidemiology of chicken anemia virus in commercial farms in China. *Virol J.* **8**, 145 (2011).
20. Renshaw, R. W. *et al.* A hypervariable region in VP1 of chicken infectious anemia virus mediates rate of spread and cell tropism in tissue culture. *J Virol.* **70**, 8872–8878 (1996).
21. Aaskov, J. *et al.* Multiple recombinant dengue type 1 viruses in an isolate from a dengue patient. *J Gen Virol.* **88**, 3334–3340 (2007).
22. Worobey, M., Rambaut, A. & Holmes, E. C. Widespread intra-serotype recombination in natural populations of dengue virus. *Proc Natl Acad Sci U S A.* **96**, 7352–7357 (1999).
23. He, C. Q. *et al.* Identification of chicken anemia virus putative intergenotype recombinants. *Virology.* **366**, 1–7 (2007).
24. Eltahir, Y. M., Qian, K., Jin, W. & Qin, A. Analysis ofchicken anemiavirusgenome: evidence of intersubtype recombination. *Virol J.* **8**, 512 (2011).
25. Yamaguchi, S. *et al.* Identification of a genetic determinant of pathogenicity in chicken anaemia virus. *J Gen Virol.* **82**, 1233–1238 (2001).
26. Simionatto, S., Lima-Rosa, C. A., Binneck, E., Ravazzolo, A. P. & Canal, C. W. Characterization and phylogenetic analysis of Brazilian chicken anaemia virus. *Virus Genes* **33**, 5–10 (2006).
27. Yuasa, N., Taniguchi, T. & Yoshida, I. Isolation and some characteristics of an agent inducing anemia in chicks. *Avian Dis.* **23**, 366–385 (1979).
28. Craig, M. I. *et al.* Molecular Characterization of Chicken Infectious Anemia Virus Circulating in Argentina During 2007. *Avian Dis.* **53**, 331–335 (2009).
29. Kim, H. R., Kwon, Y. K., Bae, Y. C., Oem, J. K. & Lee, O. S. Molecular characterization of chicken infectious anemia viruses detected from breeder and broiler chickens in South Korea. *Poult Sci.* **89**, 2426–2431 (2010).
30. Farkas, T., Tanaka, A., Kai, K. & Kanoe, M. Cloning and sequencing of the genome of chicken anaemia virus (CAV) TK-5803 strain and comparison with other CAV strains. *J Vet Med Sci.* **58**, 681–684 (1996).
31. Yamaguchi, S. *et al.* Identification of a genetic determinant of pathogenicity in chicken anaemia virus. *J Gen Virol.* **82**, 1233–1238 (2001).
32. Goryo, M., Suwa, T., Matsumoto, S., Umemura, T. & Itakura, C. Serial propagation and purification of chicken anaemia agent in MDCC-MSB1 cell line. *Avian Pathol.* **16**, 149–163 (1987).
33. Tamura, K. *et al.* MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol.* **28**, 2731–279 (2011).
34. Martin, D. P. Recombination detection and analysis using RDP3. *Methods Mol Biol.* **537**, 185–205 (2009).

35. Lole, K. S. *et al*. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. *J Virol.* **73**, 152–160 (1999).
36. Morel, V. *et al*. Genetic recombination of the hepatitis C virus: clinical implications. *J Viral Hepat.* **18**, 77–83 (2011).

Acknowledgments

This work was supported by grants from Guangdong Momentously Scientific and Technological Project (No. 2009B020201008), and the Strategic Cooperation Project of Guangdong Province & Chinese Academy (No. 2010B090301019).

Author contributions

X.Z. designed and conducted the experiments, analyzed the data, and wrote the manuscript; Q.X. modified the manuscript, and supervised the whole work; Y.L. conducted part of the experiments; B.W. and F.C. analyzed part of the data; B.S. and J.J. provided important suggestions; J.M. contributed through scientific discussions.

Additional information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Zhang, X.H. *et al.* Phylogenetic and molecular characterization of chicken anemia virus in southern China from 2011 to 2012. *Sci. Rep.* **3**, 3519; DOI:10.1038/srep03519 (2013).