Identified Novel Deletions in the Genomes of Avian Endogenous Retroviruses ev/J in Chicken Breeds in China

Xiongyan LIANG 1,† Lan LIU 1,† Chun FANG 1 Yufang GU 1 Tuofan LI 1 Yuying YANG 1

† These authors contributed equally to this work
1 College of Animal Science, Yangtze University, No. 88 Jingmi Rd., Jingzhou 434025, Hubei, P. R. CHINA

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Abstract
Avian leukosis virus subgroup J (ALV-J) infection can cause tumors and immunosuppression, which has made huge financial loss in the poultry industry. More and more new pathogenic avian leukemia virus, which were recombined from exogenous avian leukemia virus and endogenous retrovirus viruses were isolated from China and other regions in the world. To uncover the characteristic of the potential recombinant elements, we analyzed the genomes of avian endogenous retrovirus ev/J in ten chicken breeds in China. Six of the ten chicken breeds contained two sizes of ev/J (3.8 kb and 2.2 kb) and the other only contained 3.8 kb ev/J. The ten 3.8 kb ev/J were much closer to type I, II and III ev/J prototypes, while the six 2.2 kb ev/J were close to type IV ev/J prototype in the phylogenetic trees. Moreover, three novel deletion fragments were identified in the sixteen ev/J in chicken flocks in China, which made most of the ev/J (10/16) significantly different from the four ev/J prototypes. The emergence of these novel deletions resulted in the diversity of ev/J in chicken breeds in China, which may become the source of further recombination of avian leukosis viruses (ALVs), especially the exogenous ALV subgroup J.

Keywords: Avian endogenous retrovirus, ev/J, Avian leukosis viruses, Genome, Chicken breeds

INTRODUCTION
Avian leukosis virus (ALV) is an α-retrovirus that can be divided into at least 10 subgroups (from A to J) according to the sequence characteristics of their env genes [1]. ALVs can also be further divided into exogenous and endogenous viruses according to the transmission route. The exogenous viruses including subgroups A, B, C, D and J, which can infect chickens and cause neoplasms and immune suppression [1]. For the endogenous viruses, ALV subgroup E is the only group that exist in all chickens but exhibit no pathogenicity [3,4]. Among the exogenous viruses, ALV-J caused various oncogenic diseases and fertility decreasing, resulting in large economic losses in the poultry industry all over the world [5,6]. ALV-J was first isolated at the Compton Institute for Animal Health site by

Çin'de Tavuk Cinsleri Arasında Avian Endojen Retrovirus ev/J Genomunda Yeni Delesyonların Tespiti

Öz

Anahtar sözcükler: Avian endojen retrovirus, ev/J, Avian lökozis virus, Genom, Tavuk cinsleri
Payne et al. in 1988 and was thought to emerge through a recombination event between an exogenous ALV and the endogenous retrovirus elements designated EAV-HP (also termed ev/J) [8,9]. The elements of ev/J were considered as the original source of ALV-J, because their env genes shared more than 95% homology with prototype ALV-J strain HPRS-103, but shared low homology with other exogenous ALV subgroups [10,11].

The endogenous retrovirus ev/J is a member of endogenous avian virus (EAV) family, which fails to form an infectious viral particle but the reverse transcriptase (RT) remains functional [5,12]. The genomic characteristics of ev/J in chicken breeds in abroad were reported by Ruis et al. [11], Sacco et al. [13] and Sacco et al. [14] respectively. According to the deletion patterns in the gag and env junctions, these ev/J were divided into four ev/J prototypes, which were designated type I prototype EAV-HP1, type II prototype ev/J clone 3A, type III prototype ev/J clone 1C and type IV prototype EAV-HP clone 4-1. In 2004, Sacco et al. [16] reported a novel ev/J clone EAV-15I, which contains an env gene with more than 99% sequence identity to that of the ALV-J prototype HPRS-103. This finding provided another solid clue to demonstrate that ALV-J might come from the recombinant of exogenous ALVs and endogenous ev/J. Moreover, similar events also happened on ALV subgroup K, which was recently isolated from chicken in China [15-17]. These results indicated that novel recombinant elements might arise in the chicken flocks in China.

We previously reported the prevalence of endogenous retrovirus elements in chicken flocks in China [18]. But the molecular characteristics of ev/J in the chicken breeds in China remained unclear. To explore the patterns of new generated recombinant viruses, we try to analyze the genomic characteristics of ev/J from ten important chicken breeds in China. Our data demonstrated that the 3.8 kb ev/J from the ten chicken breeds in China were closely related to type I prototype EAV-HP1, type II prototype ev/J clone 3A and type III prototype ev/J clone 1C, while the 2.2 kb ev/J from six chicken breeds were close to type IV prototype EAV-HP clone 4-1. Moreover three novel deletions were found in the ev/J from tested chicken breeds. This study extends our knowledge of the molecular characteristics of ev/J in these chicken breeds.

**MATERIAL and METHODS**

**Sample Information**

Fertilized eggs of ten different chicken breeds, including Beijing fatty chicken; Shouguang chicken, Langshan chicken, Taihe chicken, Pudong chicken, Green eggshell chicken, Suqin chicken, Lohmann Brown layer, White Leghorn, and Ross Brown layer, were purchased from breeding companies in China.

**DNA Extraction**

The genomic DNA were extracted from chicken embryo fibroblasts as previously described [18]. Briefly, 10-day-old embryonated chicken eggs were minced and treated with trypsin, then the genomic DNA were extracted with Genomic DNA Extraction Kit (GK0222, Generay Biotech. Co. Ltd., Shanghai, China) according to the manufacturer's instruction. The genomic DNA samples were eluted with 50 µL of DNase-free water and stored at -80°C for using.

**PCR**

Primer pairs (forward 5‘-TTCGTGATTGGAGAAACACTTG-3‘, reverse 5‘-GTTACACCTGCGACACAAAAGGTGGCATAAC-3‘) were used to amplify the genomes of ev/J from chicken genomic DNA [11]. The PCR reaction contained 2 µL template, 5 µL 10 x buffer (Mg2+ free), 4 µL MgCl2 (25 mM), 4 µL dNTP mixture (2.5 mM each), 2.5 µL primer (10 pmol each), 0.5 µL LA Taq polymerase (DRR002A, Takara Biotechnology Co. Ltd., Dalian, China) and added DNase-free double distilled water to a total volume of 50 µL. The PCR procedure was as follows: preheating at 94°C for 5 min; then denaturing at 94°C for 30 s, annealing at indicated temperature for 30 s and extension at 72°C for 4 min for 30 cycles; final extension at 72°C for 10 min and storing at 16°C.

**DNA Cloning**

The PCR products were purified by Gel Purification kit (GK2042, Generay Biotech. Co. Ltd.) according to the manufacturer’s instructions. Purified fragments were ligated to pMD19-T vector (D102A, Takara Biotechnology Co. Ltd.) at 16°C for 4 h. The ligation products were transformed into *Escherichia coli* DH5α competent cells and plated on LB agar containing 50 µg/mL ampicillin. Positive clones were verified by PCR.

**Sequencing and Sequence Alignment**

For each sample, at least three positive clones were sequenced to ensure the identity of the sequence. The sequences were submitted to GenBank to get accession numbers. The genomic sequences of type I prototype EAV-HP 1, type II prototype ev/J clone 3A, type III prototype ev/J clone 1C and type IV prototype EAV-HP clone 4-1 were downloaded from GenBank with the accession numbers AJ238124, AF125529, AF125527 and AF125528, respectively. Then the genomic sequences were analyzed using the Clustal W method of DNAStar to determine nucleotide homology and phylogenetic trees.

**RESULTS**

Two size of fragments (3.8 kb and 2.2 kb) were amplified from the embryonic DNA of Beijing fatty chicken, Ross Brown layers, Langshan chicken, White Leghorn, Lohmann Brown layers and Shouguang chicken, while only the 3.8
kb fragment was amplified from Taihe chicken, Suqin chicken, Green eggshell chicken and Pudong chicken. Genomes of the sixteen ev/J from indicated chicken breeds were submitted to GenBank and assigned genomic accession numbers from KY085945 to KY085960 (Table 1). Phylogenetic analysis indicated that the ten 3.8 kb ev/J were highly conserved and much closer to type I, II and III ev/J prototypes, EAV-HP 1, ev/J clone 3A and ev/J clone 1C, respectively (Fig. 1a). The 2.2 kb ev/J from six chicken breeds were also conserved and were located in the same branch as EAV-HP clone 4-1 in the phylogenetic tree (Fig. 1b).

To clarify the characteristics of ev/J in the ten chicken breeds in China, we further analyzed the deletion regions

<table>
<thead>
<tr>
<th>Chicken Species</th>
<th>3.8 kb ev/J</th>
<th>2.2 kb ev/J</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Accession No.</td>
<td>Region 1</td>
</tr>
<tr>
<td>Pudong chicken (PD)</td>
<td>KY085955</td>
<td>I</td>
</tr>
<tr>
<td>Green eggshell chicken (GS)</td>
<td>KY085952</td>
<td>I</td>
</tr>
<tr>
<td>Taihe chicken (TH)</td>
<td>KY085959</td>
<td>I</td>
</tr>
<tr>
<td>Shouguang chicken (SG)</td>
<td>KY085957</td>
<td>I</td>
</tr>
<tr>
<td>White Leghorn (WL)</td>
<td>KY085960</td>
<td>II</td>
</tr>
<tr>
<td>Ross Brown layer (RB)</td>
<td>KY085956</td>
<td>III</td>
</tr>
<tr>
<td>Beijing fatty chicken (BF)</td>
<td>KY085951</td>
<td>III</td>
</tr>
<tr>
<td>Langshan chicken (LS)</td>
<td>KY085954</td>
<td>III</td>
</tr>
<tr>
<td>Lohmann Brown layer (LB)</td>
<td>KY085953</td>
<td>III</td>
</tr>
<tr>
<td>Suqin chicken (SQ)</td>
<td>KY085958</td>
<td>III</td>
</tr>
</tbody>
</table>

*Region 1 and Region 2 indicate that deletions in the gag-env and gag in the 3.8 kb ev/J, while Region 3 indicates deletion in the 3’terminal in the 2.2 kb ev/J. *I, II, III and IV indicate deletion patterns match that in type I, II, III and IV ev/J prototype, respectively; *SD1, SD2 and SD3 indicate three novel specific deletions fragments in the ev/J in chicken breeds in China.
Novel Deletions of ev/J

of ev/J in the ten chicken breeds. Comparing with the ev/J prototypes, the 3.8 kb ev/J in ten chicken breeds in China exhibited significantly different deletion pattern in two regions. The first region was located in gag-env, in which the four ev/J prototypes were designated based on the deletion patterns. According to the deletion patterns, the 3.8 kb ev/J in the ten chicken breeds can be divided into three groups. The first group including ev/J in Green eggshell chicken, Pudong chicken, Shouguang chicken and Taihe chicken, which containing the same deletion pattern as type I prototype EAV-HP1 (Table 1 & Fig. 2). Only White Leghorn contained the same deletion region as type II prototype ev/J clone 3A (Table 1 & Fig. 3). While, ev/J in other five chicken breeds including Beijing fatty chicken, Langshan chicken, Lohmann Brown layers, Ross Brown layers and Suzin chicken were grouped into type III prototype ev/J clone 1C, which containing the same deletion pattern (Table 1 & Fig. 4). In the second deletion region of 3.8 kb ev/J, which was located in the Matrix protein encoding gene gag, there were also containing three kinds of deletion among the ev/J in ten chicken breeds in China (Table 1 & Fig. 5). Deletion in the ev/J from Beijing fatty chicken, Langshan chicken, Lohmann Brown layers, Ross Brown layers and Suzin chicken was identical to that of type I and/or II prototypes ev/J. While gag region deletion of ev/J from the other five chicken breeds were quite different from that of the four ev/J prototypes, which were designated as two novel specific deletions (designated as SD1 and SD2). The ev/J from Pudong chicken and Green eggshell chicken shared SD1
and ev/J from Ross Brown layers, White Leghorn and Taihe chicken contained SD2 (Table 1 & Fig. 5). Although the 2.2 kb ev/J in the six chicken breeds were quite conserved and close to type IV ev/J prototype EAV-HP clone 4-1, a 64-bp specific deletion (designated as SD3) was observed in ev/J from Ross Brown layers (Table 1 & Fig. 6).

DISCUSSION

ALV-J, the major causative agent of avian leukosis has caused huge economic losses in the poultry industry worldwide. The emergence of ALV-J was thought to result from the recombination of an exogenous ALVs and the endogenous retroviruses EAV-HP (also termed ev/J) [8]. The proviruses of ev/J have been identified previously and divided into four typical prototypes [8,11]. The genomic size of type I, II and III ev/J prototypes were about 3.8 kb, while the genomic size type IV ev/J prototype was just 2.2 kb. In this study, we found that type I, II and III ev/J could co-exist with type IV ev/J in different chicken breeds respectively (Table 1), which were consist with previous report [14]. Although new prototypes such as EAV-15I, EAV-0, EAV-E51 and EAV-E33 were discovered more recently [8,19], the ev/J from the ten chicken breeds in China were much more closer to type I, II, III and IV ev/J prototypes. Moreover, nucleotide identity and phylogenetic tree analysis base on env gene showed that all of the ev/J were much closer to the ALV-J prototype strain HPRS-103 than other ALV-J epidemic strains isolated from China. This suggested that the endogenous retrovirus ev/J in the tested chicken breeds in China possessed a common ancestor and a similar evolutionary pathway. It also demonstrated that the exogenous ALVs that infected these chickens were more closely related to ALV-J prototype strain HPRS-103, and were different to the other epidemic strains in China [20].

In additional, we found two specific deletions (SD1 and SD2) in 3.8 kb ev/J and a 64-bp specific deletion (SD3) in 2.2 kb ev/J for the first time. These three novel deletions made ten of the sixteen ev/J from chicken breeds in China were significant different from the four ev/J prototypes. These big differences might result from the rapid evolution of ev/J in chicken [21], which might help us to and predict new recombinants of ALVs with various tumorigenesis. SD1 was located in the gag gene region of the 3.8 kb ev/J from Pudong chicken and Green eggshell chicken, and SD2 was also located in the gag gene of 3.8 kb ev/J from Ross Brown layers, White Leghorn and Taihe chicken. While SD3 was located in the 3' terminal of 2.2 kb ev/J from Ross Brown layers. As gag gene was much more conserved than env gene, these novel specific deletion might as marker to survey the recombination and evolution of both endogenous and exogenous ALVs.

In summary, we provided the novel deletion patterns in the avian endogenous retrovirus ev/J from chicken breeds in China. Further surveillance and studies need to be conducted to determine relationship of these ev/J contain novel deletion with the newly isolated pathogenic ALVs.

STATEMENT OF AUTHOR CONTRIBUTIONS

LL and XL designed and conducted experiments, analyzed data, and they were contributed equally to the work. CF analyzed data and drafted the manuscript. YG and TL were involved in study design and data collection. YY designed the entire experiments, supervised and funded the study and contributed to data analysis and to the writing of the manuscript.

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COMPLIANCE WITH ETHICAL STANDARDS

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REFERENCES