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Research Article

Identified Novel Deletions in the Genomes of Avian Endogenous Retroviruses *ev*/J in Chicken Breeds in 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 leukosis virus, which were recombined from exogenous avian leukosis 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*

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

Öz

Avian lökozis virus subgroup J (ALV-J) enfeksiyonu tümör ve immunosuprasyona neden olarak kanatlı sektöründe önemli finansal kayıpların oluşmasına yol açabilir. Eksojen avian lökozis virus ve endojen retroviruslardan rekombine edilen yeni patojenik avian lökozis viruslar her gün Çin ve dünyanın diğer bölgelerinden izole edilmektedir. Potansiyel rekombinant elementlerin özelliklerini açığa çıkarmak amacıyla Çin'de on farklı tavuk cinsinden avian endojen retrovirus *ev*/J'nin genomları analiz edildi. On cinsten altısında iki farklı *ev*/J büyüklüğü (3.8 kb ve 2.2 kb) tespit edilirken diğerlerinde sadece 3.8 kb *ev*/J mevcuttu. Filogenetik ağaçta on 3.8 kb *ev*/J tip I, II ve III *ev*/J prototiplerine çok daha yakın iken altı 2.2 kb *ev*/J ise tip IV *ev*/J prototipine yakındı. Çin'de on altı tavuk sürüsünde *ev*/J'de üç yeni delesyon parçacıkları belirlendi. Bu durum çoğu *ev*/J (10/16)'yi anlamlı derecede dört *ev*/J prototipinden farklı kılmaktadır. Bu yeni delesyonların meydana gelmesi Çin'de tavuk cinsleri arasında *ev*/J'de farklılığın oluşmasına neden olarak özellikle eksojen ALV subgrup J olmak üzere avian lökozis viruslarda ileri rekombinasyonların kaynağını oluşturabilir.

Anahtar sözcükler: *Avian endojen retrovirus, ev/J, Avian lökozis virus, Genom, Tavuk cinsleri*

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 [2]. 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

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Payne et al.[7] 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 paterns 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.[8] reported a novel *ev*/J clone EAV-15I, which contains a *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'-TTCGTGATTGGAGGAAACACTTG-3, reverse 5'-GTTACACTTGGCACACAAAGGTGGCATAAC-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 × buffer (Mg^{2+} free), 4 µL $MgCl_2$ (25 mM), 4 µL dNTP mixture (2.5 mM each), 2.5 µL primer (10 pmol each), 0.5 µL LATaq 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 1. *Deletion patterns in the ev/J in the ten chicken breeds in China*

Chicken Species	3.8 kb *ev*/J			2.2 kb *ev*/J	
	Accession No.	Region 1[a]	Region 2	Accession No.	Region 3
Pudong chicken (PD)	KY085955	I[b]	SD1[c]		
Green eggshell chicken (GS)	KY085952	I	SD1		
Taihe chicken (TH)	KY085959	I	SD2		
Shouguang chicken (SG)	KY085957	I	I/II	KY085949	IV
White Leghorn (WL)	KY085960	II	SD2	KY085950	IV
Ross Brown layer (RB)	KY085956	III	SD2	KY085948	SD3
Beijing fatty chicken (BF)	KY085951	III	I/II	KY085945	IV
Langshan chicken (LS)	KY085954	III	I/II	KY085947	IV
Lohmann Brown layer (LB)	KY085953	III	I/II	KY085946	IV
Suqin chicken (SQ)	KY085958	III	I/II		

[a] 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; [b] I, II, III and IV indicate deletion patterns match that in type I, II, III and IV ev/J prototype, respectively; [c] SD1, SD2 and SD3 indicate three novel specific deletions fragments in the ev/J in chicken breeds in China

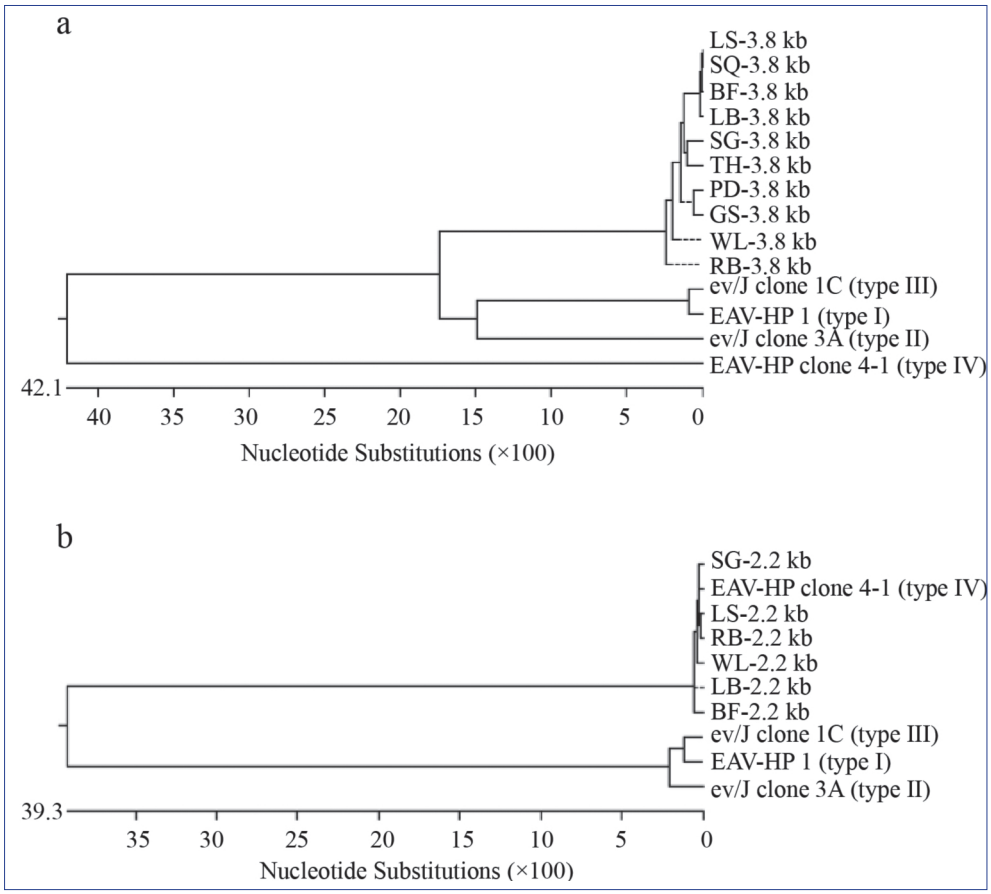

Fig 1. Phylogenetic trees base on the genomes of the 3.8 kb (a) and 2.2 kb (b) avian endogenous retroviruses were drawn by MegAlign (DNAStar)

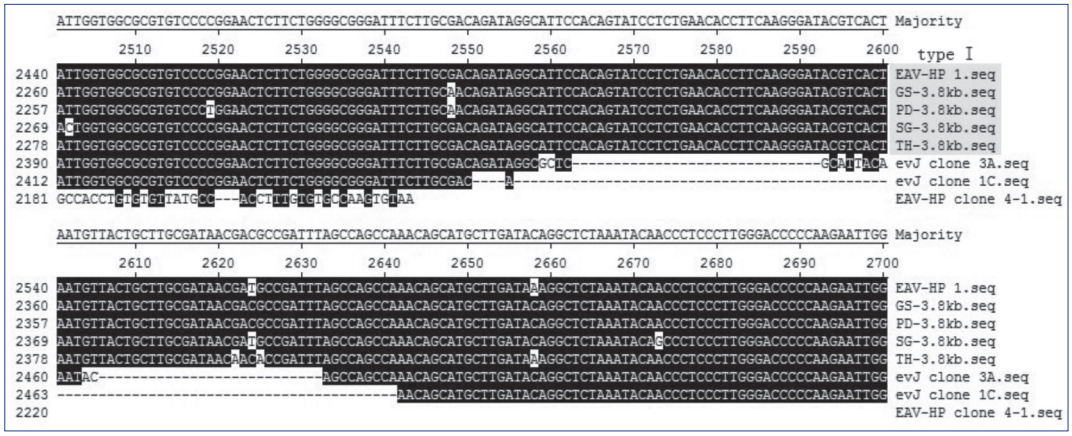

Fig 2. The 3.8 kb *ev*/J from Green eggshell chicken, Pudong chicken, Shouguang chicken and Taihe chicken contained the same deletion pattern as type I prototype EAV-HP1 in the *gag-env* region

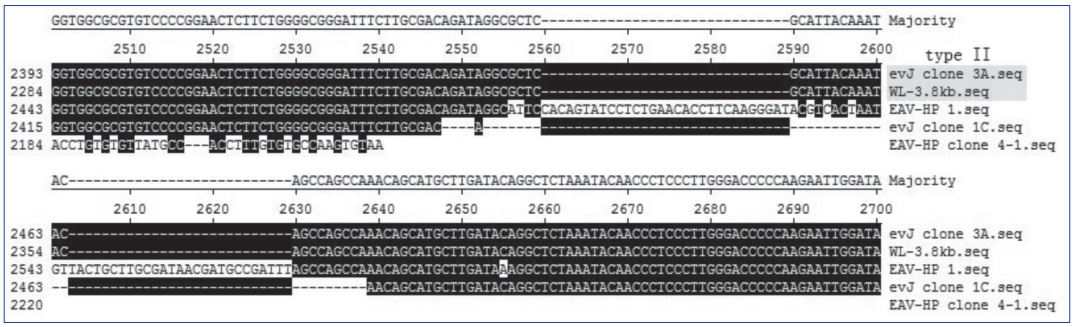

Fig 3. The 3.8 kb *ev*/J from White Leghorn contained the same deletion pattern as type II prototype *ev*/J clone 3A in the *gag-env* region

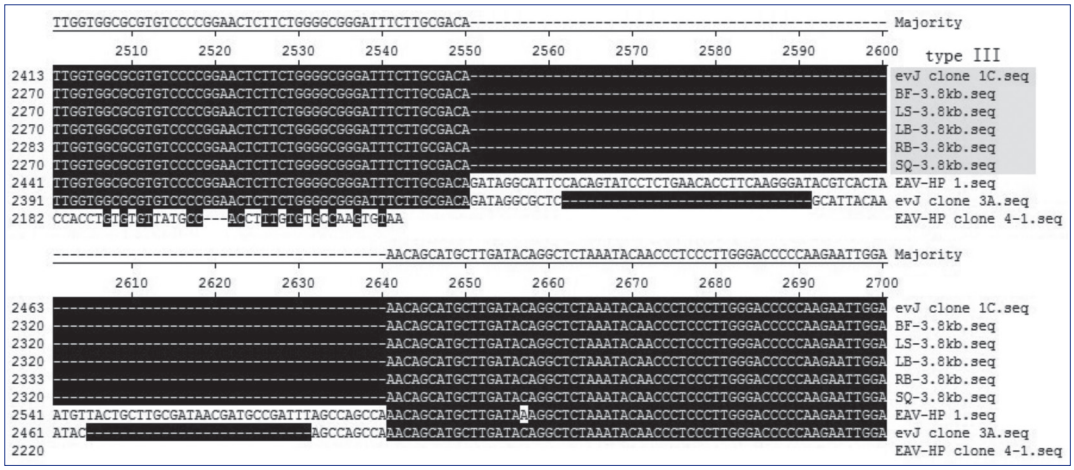

Fig 4. The 3.8 kb *ev*/J from Beijing fatty chicken, Langshan chicken, Lohmann Brown layers, Ross Brown layers and Suqin chicken contained the same deletion pattern as type III prototype *ev*/J clone 1C in the *gag-env* region

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 base 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 Suqin 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, Shouguang chicken and Suqin 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

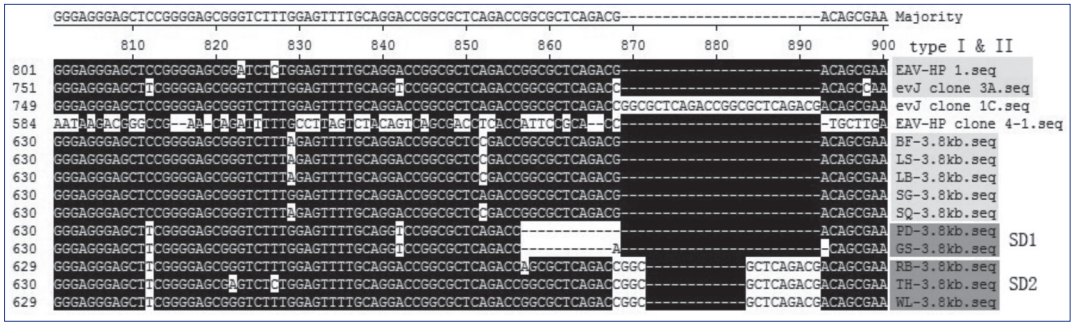

Fig 5. Deletion patterns in the the *gag* gene region of the 3.8 kb *ev*/J from the ten chicken breeds and the four ev/J prototypes

Fig 6. Deletion patterns of the 2.2 kb *ev*/J from the six chicken breeds and the four ev/J prototypes

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 of 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

1. Disclosure of potential conflicts of interest: All authors declare no conflict of interest.

2. Research involving Human Participants and/or Animals: This article does not contain any studies with human participants or animals performed by any of the authors.

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