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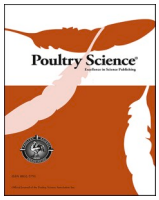

Insertion/deletion mutations within *tva* receptor gene confer chicken resistance to infection by avian leukosis virus subgroups A and K

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ABSTRACT

The classic subgroup A (ALV-A) and newly emerging subgroup K (ALV-K) of avian leukosis virus are two major pathogens responsible for avian leukemia in chickens, posing substantial threats to global poultry industry. Both viruses share a Tva protein encoded by the *tva* gene as a receptor to gain the entry into the host cells. In this study, we described the identifications of two alleles of the *tva* receptor gene in Qingyuan partridge chicken, which possesses an 11-nucleotide (GCTGCCCACCC) insertion and a 6-nucleotide (ACCTCC) deletion independently located in exon 1 of the *tva* receptor gene. The natural 11-nucleotide insertion causes a frameshift in the reading frame of the *tva* cDNA, which presumably blocks the expression of the normal *tva* allele and results resistance in chicken against infection by ALV-A and ALV-K. The natural 6-nucleotide deletion leads to a Tva receptor protein missing the amino acids residues T21 and S22, which appeared dysfunctional to mediate the viral entry. As a result, we observed that the deletion mutation in the *tva* receptor gene significantly reduced the susceptibility to infection by ALV-A and ALV-K *in vitro* and *in vivo*, and significantly reduced the binding capacity of the Tva receptor protein to the envelope glycoproteins of ALV-A and ALV-K in our subsequent analysis. Taken together, these findings not only provide evidence that the insertion and deletion mutations within the *tva* receptor gene confer chicken resistance to infection by ALV-A and ALV-K but also provide ideal targets for selective breeding of ALV-A and ALV-K resistance in chicken.

Introduction

Avian leukosis viruses (ALVs) belong to a family of pathogenic avian retroviruses that are responsible for various pathotypes of neoplastic diseases in poultry (Fandiño et al., 2023). According to the envelope antigenicity, host range and serological cross-reaction, ALVs in chickens have traditionally been classified into seven phylogenetically related subgroups, A to E, J and K (Payne and Nair, 2012; Li et al., 2021). Among these, ALV-A, ALV-B, ALV-J, and ALV-K are important pathogens of concern to poultry industry worldwide (Zhang et al., 2019, 2024; Li et al., 2021a; Lei et al., 2024). Until now, no effective vaccines or antiviral drugs are available for the control of ALVs infection. Although eradication measures have been carried out, ALVs remain to be a major enzootic challenge for the poultry industry, particularly in China and Southeast Asia (Zhang et al., 2024; Tan et al., 2024; Lei et al., 2024). Therefore, an alternative strategy to prevent and control the ALVs infection is urgently needed. In fact, selective breeding for chickens resistance to ALVs infection has proven to be an effective method to control avian leukosis (Bacon et al., 2000; Davies et al., 2009).

ALVs require interactions between the viral envelope proteins and specific cellular receptor proteins for entering the host cell, which is the first and key step in ALVs infection (Barnard and Young, 2003; Barnard et al., 2006). For example, Tva protein encoded by the *tva* gene, serves as the receptor for ALV-A (Bates et al., 1993). The *tva* gene in chicken

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determines the susceptibility or the resistance to infection by ALV-A (Young et al., 1993; Elleder et al., 2004). The decreased susceptibility or complete resistance of chickens to ALV-A can be caused by genetic mutations of the *tva* gene, leading to the expression of an aberrant Tva protein or blocks expression of the Tva protein, which are responsible for significantly reduce or even abrogate the binding affinity between ALV-A glycoproteins and Tva receptor protein (Elleder et al., 2004; Reinišová et al., 2012). Indeed, 6 *tva* resistant alleles, including tva^{r1} (Cys40Trp substitution), tva^{r2} (4-bp insertion in exon 1), tva^{r3}, tva^{r4}, tva^{r5}, and tva^{r6} (polymorphic deletions in intron 1), conferring chickens resistance to ALV-A infection have been identified in several White Leghorns inbred lines and some Chinese broiler lines (Elleder et al., 2004; Reinišová et al., 2012; Chen et al., 2017). It is noteworthy that these *tva* resistant alleles have emerged as a new object of attention owing to their ability to be used as genetic marks for the selective breeding for chicken resistance to infection by ALV-A.

ALV-K is a newly emerging ALV subgroup isolated from Chinese native chickens (Cui et al., 2014). Recent molecular epidemiologic investigations have shown that ALV-K has been prevalent and widespread among domestic chickens, which poses formidable challenges to the prevention and control of avian leukosis in China (Zhang et al., 2024; Tan et al., 2024). Notably, recent study has provided evidence that ALV-K shares the same Tva protein with ALV-A as its cellular receptor (Přikryl et al., 2019). Therefore, the *tva* alleles that confer chicken resistance to infection by both ALV-A and ALV-K is urgently needed to be explored. In the current study, we have identified two novel molecular defects, including a 11-nucleotide insertion (*c.58_59insGCTGCCCACCC*, named the $tva^{insGCTGCCCACCC}$ allele) and a 6-nucleotide deletion (*c.61_66delACCTCC*, named the $tva^{delACCTCC}$ allele), within exon 1 of the *tva* gene in Chinese chickens. These naturally occurring insertion/deletion mutations in the *tva* receptor gene confer chicken resistance to infection by ALV-A and ALV-K.

Materials And Methods

Ethics Statement

All animal experiments were approved by the Animal Ethics Committee of South China Agricultural University (**SCAU**), Guangzhou, China (approval ID: 2021b020). All the animal procedures were carried out following the recommendations in the "Guidelines for the Care and Use of Laboratory Animals" by the Animal Ethics Committee of SCAU.

Experimental Animals

The $tva^{insGCTGCCCACCC}$ allele and $tva^{delACCTCC}$ allele were separately present in Qingyuan partridge chicken (QYPC) lines F and K based on our previous study (Liao et al., 2014). QYPC lines F and K have been maintained at Guangdong Love-health Agriculture Group Limited, Guangdong, P. R. China. Cockerels and hens of QYPC lines F and K were kept separately in individual cages under standard conditions. Incubation of fertilized eggs was performed at 38°C and 55% relative humidity in an incubator.

Amplification and Analysis of tva Alleles from Qingyuan Partridge Chicken

Genomic DNA was extracted from blood samples collected from QYPC lines F and K by using DNA extraction kit (Tiangen Biotech, Beijing, China). The whole genomic region of *tva* receptor gene from DNA of lines F and K chickens was PCR amplified using four specific primer pairs (Table S1). The PCR products were directly sequenced by Sangon Biotech Co., Ltd. (Guangzhou, China). The genotypes of the *tva* alleles in each DNA samples were analyzed and confirmed. Total RNAs from livers of $tva^{s/s}$, $tva^{s/insGCTGCCCACCC}$, $tva^{insGCTGCCCACCC/insGCTGCCCACCC}$, $tva^{s/delACCTCC}$, and $tva^{delACCTCC/delACCTCC}$ chickens were isolated by TRIZOL reagent (Invitrogen) and subjected to cDNA synthesis by using PrimeScript RT Reagent Kit (Takara) according to the manufacturer's instructions. The entire *tva* coding sequence was RT-PCR amplified using specific primers as described previously (Chen et al., 2017). The purified RT-PCR products were then cloned into the TA vector pMD19-T (TaKaRa) and analyzed by sequencing.

Cell Culture and Virus Propagation

Primary chicken embryo fibroblasts (CEFs) were prepared from 9-day-old embryos from QYPC line K. The procedure was described previously (Li et al., 2018). The genotypes of the $tva^{delACCTCC}$ allele in CEFs were determined by direct sequencing as described above. CEFs and DF-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) with 10% fetal bovine serum (FBS, Life Technologies), 100 units/ml of penicillin, and 100 mg/ml of streptomycin in a 5% CO_2 atmosphere at 39°C. The infectious virus RCASBP (A)-EGFP and RCASBP(K)-EGFP, transducing the EGFP reporter gene, was prepared by RCASBP(A)-EGFP and RCASBP(K)-EGFP plasmid DNA transfection into DF-1 cells. Transfection was performed by using Lipofectamine 3000 transfection reagent (BioSciences Ltd., Dublin, Ireland.) according to the manufacturer's instructions. On day 7 post-transfection, the virus stocks were harvested and virus titer was determined as described previously (Chen et al., 2017). The ALV-A strain GD-08 and the ALV-K strain GD-1601 were propagated in DF-1 cells. The value of the ratio of the sample to the positive control (S/P) for ALV p27 antigen of the ALV-A strain GD-08 and the ALV-K strain GD-1601 was determined by using the avian leukosis virus antigen test kit (Harbin Guosheng Biotechnology Co., LTD, China)

RCASBP(A)-EGFP and RCASBP(K)-EGFP Virus Spread Assayed by Flow Cytometry

CEFs of defined origin were mock infected or infected by RCASBP (A)-EGFP or RCASBP(K)-EGFP fluorescence reporter virus. In brief, the $tva^{s/s}$, $tva^{s/delACCTCC}$, and $tva^{delACCTCC/delACCTCC}$ genotypes CEFs were seeded in triplicate wells at a density of 5×10^4 per well in a 24-well plate, and subsequently infected with either RCASBP(A)-EGFP or RCASBP(K)EGFP at a multiplicity of infection (MOI) of 10 at 24 h after seeding. After incubation for 2 h, CEFs were overlaid with DMEM supplemented with 1% FBS and incubated at 39°C in a 5% CO_2 atmosphere for 7 days. The percentage of GFP-positive cells was quantitated by fluorescence-activated cell sorting (FACS) using a BD FACSVerse™ Flow Cytometer (Becton, Dickinson and Company) on days 1, 2, 3 and 7 post-infection. Before FACS analysis, the cells of three wells were trypsinized and washed in phosphate-buffered saline (PBS).

Susceptibility Assay of ALV-A and ALV-K by Real-time qPCR

The $tva^{s/s}$, $tva^{s/delACCTCC}$, and $tva^{delACCTCC/delACCTCC}$ CEFs were seeded in triplicate wells at a density of 5×10^5 per well in a 12-well plate. At 24 h after seeding, CEFs were infected with ALV-A strain GD-08 (S/P value, 2.2) or ALV-K strain GD-1601 (S/P value, 2.1) at a MOI of 0.1. At 2h postinfection, the medium was replaced by fresh medium, and CEFs were incubated in a 5% CO_2 atmosphere at 39°C for 4 days. At days 1, 2, 3 and 4 postinfection, the infected cell cultures were harvested and subjected to RNA extraction and cDNA synthesis. The titer of ALV-A and ALV-K were determined as the virus copies per milliliter by Real-time quantitative polymerase chain reaction (RT-qPCR) using ALV-A strain GD-08 specific primers pair (Table S1) and ALV-K strain GD-1601 specific primers pair as previously described (Chen et al., 2018), respectively.

ALV-A and ALV-K Infection Experiment in vivo

One-day-old chicks randomly collected from QYPC line K were randomly divided into four groups with 20 birds each. 3 specific

pathogen-free (SPF) chicks were used for controls in each group. Chicks of two group were inoculated with 0.4 ml of ALV-A strain GD-08 (2.2 S/P) through the abdominal cavity, and inoculated once again at 5 days old. Chicks of the other two independent group were inoculated with 0.4 ml of ALV-K strain GD-1601 (2.1 S/P) through the abdominal cavity, and inoculated once again at 5 days old. Chicks were housed in negative-pressure-filtered air isolators, feed and water were provided *ad libitum*. Genomic DNA of whole blood sample from each chick at 2 weeks old was used to genotype the *tva*$^{\text{delACCTCC}}$ allele by direct sequencing. At one month postinfection, the status of infection in each chick by ALV-A strain GD-08 and ALV-K strain GD-1601 was determined by using the method as previously described (Chen et al., 2017) and using two specific primer pairs (Table S1), respectively.

Construction and Production of Fusion Protein SU(A)-rIgG and SU(K)-rIgG

The procedure for construction of recombinant retroviral vectors RCASBP(B)-SU(A)-rIgG and RCASBP(B)-SU(K)-rIgG were similar to that described as previously (Li et al., 2018). In brief, the gp85 (SU) coding regions of ALV-A and ALV-K were amplified using RT-PCR from the ALV-A strain GD-08 and the ALV-K strain GD-1601, respectively. Similarly, the rabbit IgG heavy chain (rIgGFc) was obtained by RT-PCR from rabbit blood cell RNA. The SU(A), SU(K) fragments and the rIgGFc were ligated with SacI/KpnI (New England BioLabs) restriction enzyme sites, and the resulting fusion fragment was subcloned into the pUC18 vector (Promega, Madison, USA), to generate the pUC18-SU(A)-rIgG, pUC18-SU(K)-rIgG vectors containing a ca. 1.8kb fragment that fused ALV-A SU or ALV-K SU and rIgGFc genes, repectively. Subsequently, we cloned SU(A)-rIgG and SU(K)-rIgG into the Cla12Nco adapter plasmid using the SacI and BamHI restriction sites to generate the recombinant Cla12NcoSU(A)-rIgG and Cla12Nco-SU(K)-rIgG vectors. The SU(A)-rIgG and SU(K)-rIgG sequence were then cloned into RCASBP(B) vector as the ClaI fragment. The final recombinant vectors RCASBP(B)-SU(A)-rIgG and RCASBP(B)-SU(K)-rIgG were transfected into DF-1 cells. The cell supernatants that expressed immunoadhesin proteins SU(A)-rIgG or SU(K)-rIgG were cleared by centrifugation at 2,000 × g for 10 min at 4°C, and then stored at -80°C in aliquots. The levels of all two soluble SU proteins were quantitated by enzyme-linked immunosorbent assay (ELISA) for the rabbit IgG tag. The primers for construction of RCASBP(B)-SU(A)-rIgG and RCASBP(B)-SU(K)-rIgG vectors are listed in Table S1 in the supplemental material.

Western Immunoblot Analysis

At 48 h posttransfection, the immunoadhesin proteins SU(A)-rIgG and SU(K)-rIgG were immunoprecipitated separately from DF-1 cell culture supernatants using anti-rabbit agarose beads (Sigma), and then were analyzed by Western immunoblotting as described previously (Zingler and Young, 1996). The horseradish peroxidase-conjugated goat anti-rIgG (1:10,000; Proteintech Group, Inc., USA) was used as the secondary antibody. The SU(A)-rIgG and SU(K)-rIgG protein complexes were detected by Lightning Chemiluminescence reagent Plus (Perkin Elmer Life Sciences Inc).

Binding Affinity was Analyzed by Flow Cytometry

The *tva*$^{s/s}$, *tva*$^{s/\text{delACCTCC}}$, and *tva*$^{\text{delACCTCC/delACCTCC}}$ CEFs expressing the respective Tva receptor proteins were seeded in triplicate wells at a density of 2 × 10^5 per well in a 6-well plate. After reaching approximately 90% confluence, CEFs were harvested by 0.25% trypsin solution, and washed in PBS supplemented with 2% calf serum (PBS-2%CS), centrifuged for 5 min at 600 × g, and resuspended in 200 μL of PBS-2% CS. The cells in PBS-2%CS were then incubated with supernatant containing SU(A)-rIgG or SU(K)-rIgG fusion protein, both at a concentration of 10 ng of rIgG Fc fragment per mL, on ice for 1 hour. Following three washes with PBS-2%CS, goat anti-rabbit IgG linked to Alexa Fluor 488 (Invitrogen) was diluted 1:100 in PBS-4%CS, and the washed cells were incubated in 500 μL of the diluted antibody solution on ice for 30 minutes. After incubation and three washes in PBS-2%CS, the cells with immunoadhesin complexes were resuspended in 200 μL of PBS-4%CS, and the percentage of fluorescence-positive cells was quantified by FACS using BD FACSVerse^{TM} Flow Cytometer (Becton, Dickinson and Company).

Statistical Analysis

Statistical analysis were performed using GraphPad Prism (version 8.0) software (GraphPad Software Inc.). The ALV-A and ALV-K infections *in vitro* and binding affinity experiments were performed with at least three independent replicates, the results are presented as means ± standard error, and statistical significance was assessed at $P < 0.05$, 0.01.

Results

Identification of Novel tva Alleles in Qingyuan Partridge Chicken

To dissect genetic mutations within the *tva* receptor gene in Qingyuan partridge chicken (QYPC) lines F and K, we amplified and sequenced the whole genomic region of the *tva* gene in each chicken (Fig. S1A). Two novel *tva* allelic variants located within exon 1 of the *tva* gene were discovered in Qingyuan partridge chickens surveyed. Sequencing revealed that one new variant inserted 11-nucleotide sequence GCTGCCCACCC, while the other new variant deleted 6-nucleotide sequence ACCTCC, which were designated as *tva*$^{\text{insGCTGCCCACCC}}$ and *tva*$^{\text{delACCTCC}}$ alleles, respectively (Fig. 1A and 1B). The *tva*$^{\text{insGCTGCCCACCC}}$ and *tva*$^{\text{delACCTCC}}$ alleles were separately present in QYPC line F and QYPC line K, respectively. To further confirm the mutations in *tva*$^{\text{insGCTGCCCACCC}}$ and *tva*$^{\text{delACCTCC}}$ transcripts, we amplified the entire *tva* coding sequence from livers samples of defined origin, and RT-PCR products were then cloned sequencing (Fig. S1B). We found that both the longer and shorter *tva*$^{\text{insGCTGCCCACCC}}$ transcripts did contain the 11-nucleotide (GCTGCCCACCC) insertion located in exon 1 at nucleotide positions 58 and 59 of the *tva* cDNA (*c.58_59insGCTGCCCACCC*), while both the longer and shorter *tva*$^{\text{delACCTCC}}$ transcripts contained 6-nucleotide (ACCTCC) deletion in exon 1 at nucleotides 61 to 66 of the *tva* cDNA (*c.61_66delACCTCC*) (Fig. 1C).

Subsequently, we compared the deduced amino acid sequences of the long and short isoforms of chicken Tva originating from the *tva*^{s} allele with those corresponding to the *tva*$^{\text{insGCTGCCCACCC}}$ allele (Fig. S2). The insertion of an 11-nucleotide sequence (GCTGCCCACCC) led to the alteration of the amino acid sequence of the Tva receptor protein. Specifically, the c.58_59insGCTGCCCACCC of the *tva*$^{\text{insGCTGCCCACCC}}$ allele induces a frameshift mutation in the *tva* cDNA at the 20th codon within exon 1. This mutation also introduces a premature TGA stop codon at the 112th codon in exon 3, affecting both the long and short isoforms of the chicken Tva. As a result, a frameshifted peptide consisting of 92 residues is presumably appended to the truncated Tva receptor. This truncated receptor is missing a portion of residues in the transmembrane (TM) and cytoplasmic tail regions (Fig. 1C and 1D). When compared to the deduced amino acid sequences of both the long and short isoforms of Tva from the *tva*^{s} allele, six cysteine residues, along with the critical functional residues E53, L55, H59, and W69 in the LDL-A module, underwent substitution. This LDL-A module is responsible for binding to ALV-A and ALV-K gp85 and facilitating the entry of ALV-A and ALV-K into host cells (Přikryl et al., 2019). These substitutions occurred in the 92-residue frameshifted peptide of the truncated Tva receptor derived from the *tva*$^{\text{insGCTGCCCACCC}}$ allele, as depicted in the Fig. 1E. Consequently, it is plausible that the *tva*$^{\text{insGCTGCCCACCC}}$ allele inhibits the expression of functionally normal Tva proteins. This inhibition confers complete resistance in chickens to infection by both ALV-A and ALV-K.

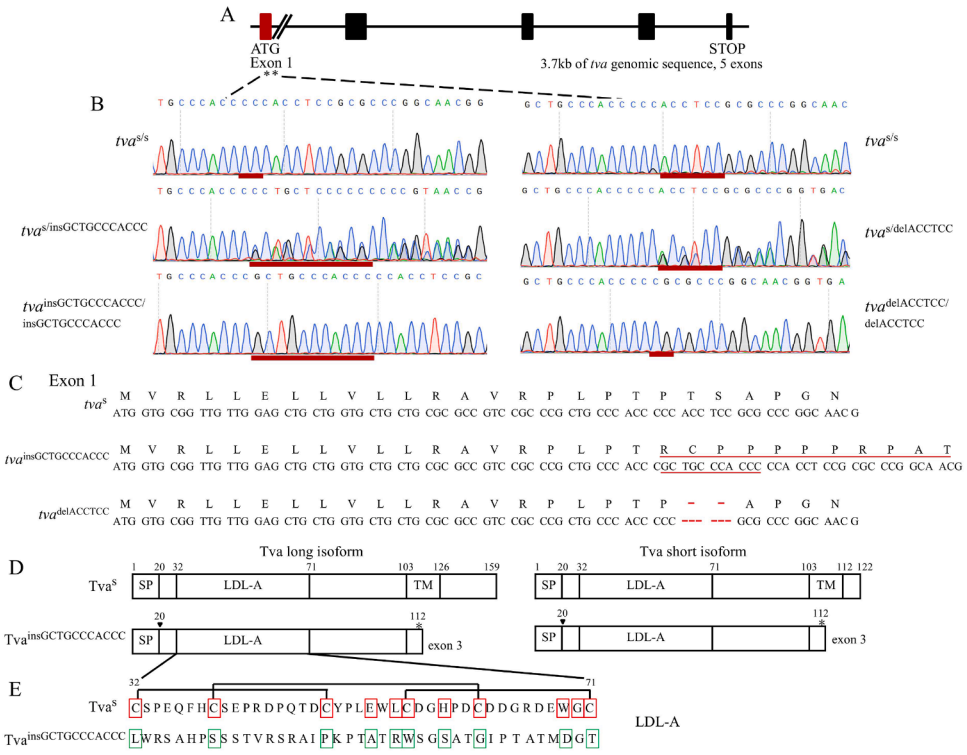

Fig. 1. Identification of the *tva*^{insGCTGCCCACCC} and *tva*^{delACCTCC} Alleles in Qingyuan partridge chicken. (A) A schematic drawing of the structure of the *tva* receptor gene. **(B)** Sequences traced of the wild-type *tva*$^{s/s}$, heterozygous *tva*$^{s/insGCTGCCCACCC}$ and homozygous *tva*$^{insGCTGCCCACCC/insGCTGCCCACCC}$ genotypes of the *tva*^{insGCTGCCCACCC} allele are showed and the 11-nucleotide (GCTGCCCACCC) insertion in the *tva*^{insGCTGCCCACCC} allele are highlighted in red line. Sequences traced of the wild-type *tva*$^{s/s}$, heterozygous *tva*$^{s/delACCTCC}$ and homozygous *tva*$^{delACCTCC/delACCTCC}$ genotypes of the *tva*^{delACCTCC} allele are showed and the 6-nucleotide (ACCTCC) deletion in the *tva*^{delACCTCC} allele are highlighted in red line. **(C)** The alignment of the nucleotide sequence and deduced amino acid sequence of exon 1 in the *tva*^{s} allele with the *tva*^{insGCTGCCCACCC} and *tva*^{delACCTCC} alleles. The 11-nucleotide insertion and altered amino acid sequence of Qingyuan partridge chicken line F are highlighted in red, the 6-nucleotide deletion and the missing amino acids residues T21 and S22 are highlighted in red dashs. **(D)** Schematic drawings of the Tva^{S} and Tva^{insGCTGCCCACCC} proteins. The numbering of the Tva^{S} protein includes the signal peptide (SP), cysteine-rich region (LDL-A), transmembrane (TM) and cytoplasmic tail regions. The positions of the frameshift mutations in Tva^{insGCTGCCCACCC} are highlighted with a triangle, while the premature TGA stop codons in Tva^{insGCTGCCCACCC} is indicated by an asterisk. **(E)** Comparison of the deduced amino acid sequences of the LDL-A of the chicken Tva receptors. The putative disulfide bonds in the LDL-A of Tva^{S} are indicated. The six cysteine residues along with the critical functional residues E53, L55, H59, W69 and G70 in the LDL-A module of Tva^{S} are indicated in red squares, while substitutions of these residues in the *tva*^{insGCTGCCCACCC}-derived peptide are indicated in green squares.

Resistance of Qingyuan Partridge Chicken to RCASBP(A)-EGFP and RCASBP(K)-EGFP Infection in vitro

The *c.61_66delACCTCC* mutation in the *tva*^{delACCTCC} allele results in the deletion of threonine 21 (T21) and serine 22 (S22) residues in the Tva receptor protein (Fig. 1C). To determine the effects of the *tva*^{delACCTCC} allele on ALV-A and ALV-K susceptibility, we firstly rescued and obtained the replication-competent RCASBP(A)-EGFP and RCASBP(K)-EGFP fluorescent reporter viruses (Fig. S3). The *tva*$^{s/s}$, *tva*$^{s/delACCTCC}$, *tva*$^{delACCTCC/delACCTCC}$ CEFs were then infected with the RCASBP(A)-EGFP or RCASBP(K)-EGFP, and virus spread was followed as percentage of GFP-positive cells quantified by FACS over 7 subsequent days. Since the wild-type *tva*$^{s/s}$ CEFs are susceptible to ALV-A and ALV-K, thus were used as a positive control. Consistent with expectations, RCASBP(A)-EGFP and RCASBP(K)-EGFP infected the wild-type *tva*$^{s/s}$ CEFs very efficiently, both with an infection rate of approximately 65% on day 1, and reaching virtually complete infection of cells by day 7 (Fig. 2A and 2B). However, RCASBP(A)-EGFP and RCASBP(K)-EGFP infected the homozygous *tva*$^{delACCTCC/delACCTCC}$ CEFs are much less efficiently, with only 1%-5% of infected cells on day 1, and with only 23% and 26% of the cells being infected at 7 days post-infection, respectively (Fig. 2A and 2B). In contrast, the heterozygous *tva*$^{s/delACCTCC}$ CEFs were much highly susceptible to infection by RCASBP(A)-EGFP and RCASBP(K)-EGFP, both infecting up to a higher infection rate with over 90% on day 7 (Fig. 2A and 2B). The GFP-negative and GFP-positive cells are obviously disparate, as shown by the FACS dot-plots (Fig. 2C and 2D). These results clearly demonstrated the inefficient infection and slow spread of RCASBP(A)-EGFP and RCASBP(K)-EGFP in the homozygous *tva*$^{delACCTCC/delACCTCC}$ CEFs.

Resistance of Qingyuan Partridge Chicken to ALV-A and ALV-K Infection in vitro

To further confirm the results of RCASBP(A)-EGFP and RCASBP(K)-EGFP infection, we also infected CEFs of different genotypes at the *tva*^{delACCTCC} allele with the ALV-A strain GD-08 and the ALV-K strain GD-1601, and subsequently determined the growth kinetics of ALV-A and ALV-K in CEFs by RT-qPCR, respectively. As shown in Fig. 3A and 3B, both the ALV-A and ALV-K replicated quickly in the wild-type *tva*$^{s/s}$ CEFs, reaching a titer of 10^8 virus copies/ml by days 4 post-infection. In contrast, the ALV-A and ALV-K replicated more slowly in the *tva*$^{delACCTCC/delACCTCC}$ CEFs, and reached a titer with only 10^2 virus copies/ml by day 4. While the *tva*$^{s/delACCTCC}$ CEFs were efficiently infected by the ALV-A and ALV-K, exhibiting growth kinetics similar to those in wild-type *tva*$^{s/s}$ CEFs. Altogether, these results formally demonstrated that the 6-nucleotide (ACCTCC) deletion mutation in the *tva* receptor gene resulted in reduced susceptibility of QYPC line K to infection by ALV-A and ALV-K *in vitro*.

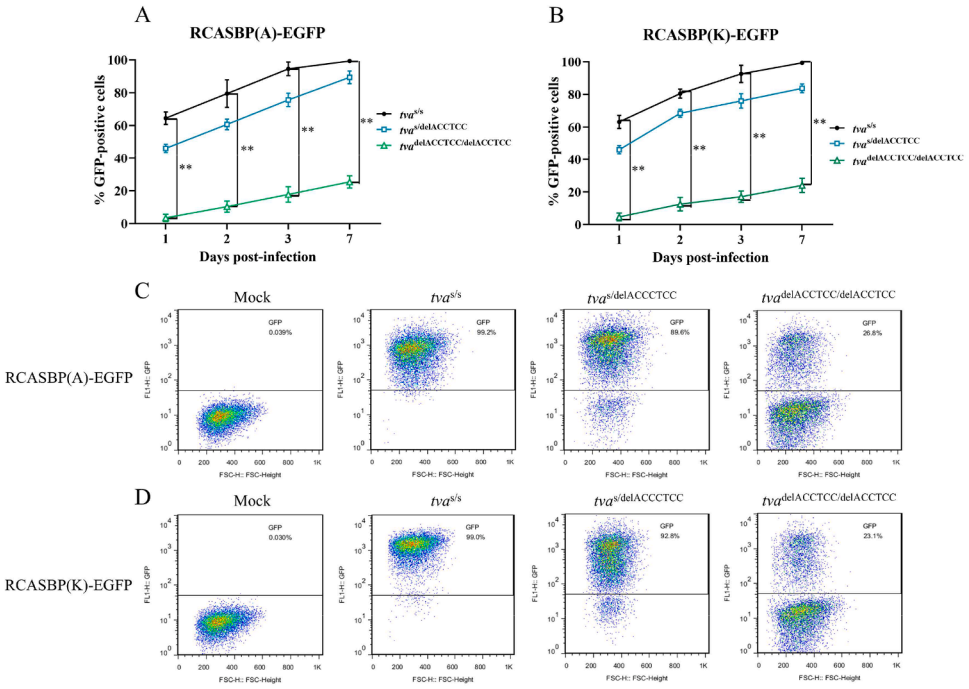

Fig. 2. Time course of infection of *tva*$^{s/s}$, *tva*$^{s/delACCTCC}$ and *tva*$^{delACCTCC/delACCTCC}$ CEFs with ALV reporter viruses. CEFs of defined origin were mock infected or infected by RCASBP(A)-EGFP or RCASBP(K)-EGFP fluorescence reporter virus at an MOI of 10. (A and B) The proportion of GFP-positive cells of the wild type *tva*$^{s/s}$, heterozygous *tva*$^{s/delACCTCC}$ and homozygous *tva*$^{delACCTCC/delACCTCC}$ genotypes CEFs was determined by FACS on indicated days postinfection, and the percentage of GFP-positive cells is indicated as a mean and standard error of mean from three parallel dishes. The statistical significance of differences in growth changes of RCASBP(A)-EGFP or RCASBP(K)-EGFP reporter viruses between the wild type *tva*$^{s/s}$ CEFs and the homozygous mutant *tva*$^{delACCTCC/delACCTCC}$ CEFs at different time points tested is indicated (**, $P< 0.01$). **(C)** Representative FACS dot-plots of CEFs of mock-infected and CEFs of different genotypes for the *tva*^{delACCTCC} allele infected with RCASBP(A)-GFP at 7 days postinfection. **(D)** Representative FACS dot-plots of CEFs of mock-infected and CEFs of different genotypes for the *tva*^{delACCTCC} allele infected with RCASBP(K)-GFP at 7 days postinfection. The relative GFP fluorescence is plotted against the cell count, and the percentage of GFP-positive cells is indicated.

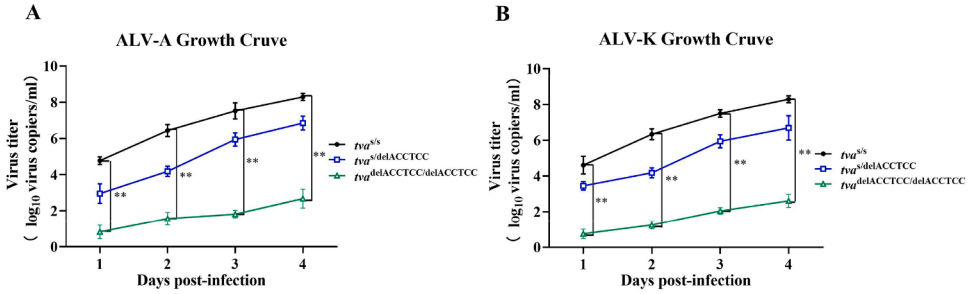

Fig. 3. Growth curves of ALV-A and ALV-K in CEFs of defined origin. The *tva*$^{s/s}$, *tva*$^{s/delACCTCC}$, and *tva*$^{delACCTCC/delACCTCC}$ CEFs were infected with ALV-A strain GD-08 or ALV-K strain GD-1601 at a MOI of 0.1. Cell culture supernatant-associated viruses were harvested at 1, 2, 3 and 4 days postinfection, and titration results were determined as virus copies using RT-qPCR. Means±SD of data from three independent experiments performed in triplicates are shown. **(A)** ALV-A strain GD-08 growth curves in the *tva*$^{s/s}$, *tva*$^{s/delACCTCC}$, and *tva*$^{delACCTCC/delACCTCC}$ CEFs. (B) ALV-K strain GD-1601 growth curves in the *tva*$^{s/s}$, *tva*$^{s/delACCTCC}$, and *tva*$^{delACCTCC/delACCTCC}$ CEFs. The statistical significance of differences in virus copies of ALV-A strain GD-08 or ALV-K strain GD-1601 between the wild type *tva*$^{s/s}$ CEFs and the homozygous mutant *tva*$^{delACCTCC/delACCTCC}$ CEFs at different time points tested is indicated (**, $P< 0.01$).

Resistance of Qingyuan Partridge Chicks to ALV-A and ALV-K Infection in vivo

In order to further determine the effects of the *tva*^{delACCTCC} allele on the infection and replication of the ALV-A and ALV-K *in vivo*, 1-day-old chicks of defined origin were inoculated with either ALV-A strain GD-08 or ALV-K strain GD-1601 through the abdominal cavity. At one month post-infection, the infection status of ALV-A or ALV-K was determined by RT-PCR of RNA extracted from whole blood samples of each chick. As shown in Table 1, 6 SPF chicks used as positive controls were all positive for ALV-A, confirming that the challenge trial assay was successful. As anticipated, all of the 17 wild-type chicks were positive for ALV-A. In the cohort of 14 heterozygous *tva*$^{s/delACCTCC}$ chicks, 11 chicks were positive for ALV-A. In contrast, only 1 out of 9 homozygous *tva*$^{delACCTCC/delACCTCC}$ chicks were positive for ALV-A. In the other independent challenge experiment, both the SPF chicks (positive controls) and wild-type *tva*$^{s/s}$ chicks were all positive for ALV-K (Table 2). In the cohort of 13 *tva*$^{s/delACCTCC}$ chicks, except for 2 chicks, were also positive for ALV-K. However, the susceptibility to ALV-K of the homozygous *tva*$^{delACCTCC/delACCTCC}$ chicks decreased. In the cohort of 15 *tva*$^{delACCTCC/delACCTCC}$ chicks, only 2 chicks were positive for ALV-K (Table 2). Taken together, the *in vivo* infection corresponds with the spread of the virus *in vitro* and confirms the reduced susceptibility of QYPC line K to both ALV-A and ALV-K.

Table 1
Incidence of infection by ALV subgroup A.

Chicks	Genotypes[2]	No. of positive samples/total no. of samples	Positive infection (%)
SPF	$tva^{s/s}$	6/6	100
QYPC[1] line K	$tva^{s/s}$	17/17	100
	$tva^{s/delACCTCC}$	11/14	78.6
	$tva^{delACCTCC/delACCTCC}$	1/9	11.1

[1] QYPC= Qingyuan partridge chicken
[2] $tva^{s/s}$ = susceptible homozygote of *tva* receptor gene (wild genotype); $tva^{s/delACCTCC}$=heterozygote of deleted ACCTCC; $tva^{delACCTCC/delACCTCC}$=homozygote of deleted ACCTCC.

Table 2
Incidence of infection by ALV subgroup K.

Chicks	Genotypes	No. of positive samples/total no. of samples	Positive infection (%)
SPF	$tva^{s/s}$	6/6	100
QYPC[1] line K	$tva^{s/s}$	12/12	100
	$tva^{s/delACCTCC}$	11/13	84.6
	$tva^{delACCTCC/delACCTCC}$	2/15	13.3

[1] QYPC= Qingyuan partridge chicken
[2] $tva^{s/s}$ = susceptible homozygote of *tva* receptor gene (wild genotype); $tva^{s/delACCTCC}$=heterozygote of deleted ACCTCC; $tva^{delACCTCC/delACCTCC}$=homozygote of deleted ACCTCC.

The Deletion Mutation within the tva Gene Decreases the Binding Affinity for the ALV-A and ALV-K Envelope Glycoproteins

To further determine the effects of deletion mutation within the *tva* gene on the binding affinity of the Tva receptor proteins with the ALV-A and ALV-K glycoproteins, we constructed chimeric immunoadhesins SU (A)-rIgG and SU(K)-rIgG (Fig. 4A). These immunoadhesins consist of the subgroup A or K-specific surface (SU) glycoproteins fused to the constant fragment of rabbit IgG, and were transfected into DF-1 cells. The expression of fusion proteins SU(A)-rIgG or SU(K)-rIgG was detected and verified by Western immunoblot analysis (Fig. 4B). The binding capacity of SU(A)-rIgG or SU(K)-rIgG to Tva receptor proteins expressed on the surfaces of $tva^{s/s}$, $tva^{s/delACCTCC}$ and $tva^{delACCTCC/delACCTCC}$ CEFs was detected using fluorescein-conjugated goat anti-rabbit antibodies and quantified by flow cytometry. The percentage of fluorescein-positive cells reflects the binding capacity of viral glycoproteins-Tva receptor protein. As shown in Fig. 4C and 4D, the binding capacity of SU(A)-rIgG or SU(K)-rIgG fusion proteins with the Tva receptor protein expressed on the surfaces of $tva^{delACCTCC/delACCTCC}$ CEFs was significantly lower than that with Tva receptor protein expressed in the wild type $tva^{s/s}$ CEFs ($P<0.01$), respectively. The Alexa Fluor-488 fluorescein-negative and fluorescein-positive cells are clearly different, as indicated by the presence of two separate peaks in the FACS histograms (Fig. 4E). Collectively, these findings demonstrated that the 6-nucleotide (ACCTCC) deletion mutation in the *tva* gene caused dramatically reduces the binding affinity for ALV-A and ALV-K envelope glycoproteins with Tva receptor protein.

Discussion

ALV-A and ALV-K both cause tumor-induced mortality, growth retardation and severe immunosuppression in chickens (Chen et al., 2022). Recent epidemiological investigations have indicated that both ALV-A and ALV-K are quite widespread among domestic chickens and have become important concerns for the poultry industry, especially in China and Southeast Asia (Dou et al., 2023; Tan et al., 2024; Zhang et al., 2024). Thus, the prevalence of ALV-A and ALV-K in chicken populations might have imposed chickens strong selection pressure to develop complete or partial resistance to ALV-A and ALV-K infection. In this study, we have identified two molecular defects, including 11-nucleotide (GCTGCCCACCC) insertion ($tva^{insGCTGCCCACCC}$ allele) and 6-nucleotide (ACCTCC) deletion ($tva^{delACCTCC}$ allele), both located in exon 1 of the *tva* receptor gene in Qingyuan partridge chicken lines. The $tva^{insGCTGCCCACCC}$ allele presumably confers chickens complete resistance to infection by ALV-A and ALV-K, while the $tva^{delACCTCC}$ allele confers chickens significantly reduce the susceptibility to infection by both ALV-A and ALV-K, which was confirmed both *in vitro* and *in vivo*. The 6-nucleotide (ACCTCC) deletion in Qingyuan partridge chicken line K significantly reduces the binding affinity of the Tva receptor for the ALV-A and ALV-K envelope glycoproteins and account for the decreased infection efficiency of ALV-A and ALV-K. To our best of knowledge, this study is the first to report the *tva* alleles that are responsible for conferring host resistance or decreased susceptibility to infection by both ALV-A and ALV-K.

ALV-A and ALV-K share Tva protein encoded by the *tva* gene as receptor to enter host cells (Přikryl et al., 2019). Tva protein consists of an extracellular domain of 83 amino acids, a transmembrane region of 23 amino acids, and an intracellular region of 33 amino acids (Bates et al., 1993). The extracellular domain of the chicken Tva receptor showcases a single cysteine-rich segment known as the LDL-A region, which encompasses amino acid residues from 32 to 71. This area is characterized by the presence of six cysteine residues, which form three disulphide bonds. These disulphide bonds play a pivotal role in stabilizing the structure of the LDL-A region. Previous investigations have revealed that the LDL-A region of Tva has a strong affinity for the gp85 glycoprotein of both ALV-A and ALV-K (Prikryl et al., 2019). This high-affinity interaction is not only essential but also sufficient for Tva to mediate the entry of ALV-A and ALV-K into host cells (Rong et al., 1995, 1997; Prikryl et al., 2019). Recent studies have shed light on specific functional residues within Tva. Residues L55 and W69 of Tva have been identified as crucial for binding to ALV-A gp85. This interaction is what allows ALV-A to gain entry into host cells (Chen et al., 2022). In the case of ALV-K, residues E53, L55, H59, and G70 of Tva are the key players in facilitating virus entry. Any substitution of these residues disrupts the interaction between Tva and ALV-K gp85. For instance, DF-1 cells in which E53, L55, H59, and G70 of Tva have been substituted show complete resistance to ALV-K entry (Li et al., 2023). When comparison of the deduced amino acid sequences of both the long and short isoforms of Tva from the tva^{s} allele with the frameshifted 92-residue peptide of the truncated Tva receptor derived from the $tva^{insGCTGCCCACCC}$ allele reveals significant differences. The six cysteine residues, along with the critical functional residues E53, L55, H59, and W69 in the LDL-A module, are substituted in the $tva^{insGCTGCCCACCC}$-derived peptide (Fig. 1E). Based on these findings, it is reasonable to infer that the $tva^{insGCTGCCCACCC}$ allele likely hampers the expression of functionally normal Tva proteins. As a result, chickens carrying this allele could potentially be completely resistant to infection by both ALV-A and ALV-K. As a parallel to the $tva^{insGCTGCCCACCC}$ allele, the tva^{r2} allele presents in the White Leghorns inbred line 7_2 harbors a 4-bp insertion (CTCG) within exon 1 of the *tva* gene, leading to a frameshift mutation and ultimately disrupt the normal sequence of amino acids in the Tva receptor. This disruption prevents the correct folding and function of the receptor, making it ineffective in mediating ALV-A infection, which accounts for the complete resistance of Line 7_2 to infection by ALV-A (Elleder et al., 2004).

Although the underlying mechanism behind the reduced susceptibility to ALV-A and ALV-K associated with the 6-nucleotide (ACCTCC) deletion in the *tva* receptor gene remains unclear at present, it is known that this deletion event in exon 1 of the *tva* receptor gene leads to a distinct alteration in the Tva receptor protein. Specifically, the deletion results in the absence of amino acid residues T21 and S22 within the Tva receptor protein. These two residue positions are hypothesized to play a vital role in the binding affinity between the Tva receptor and the glycoproteins of ALV-A and ALV-K. The binding of a virus to its cellular

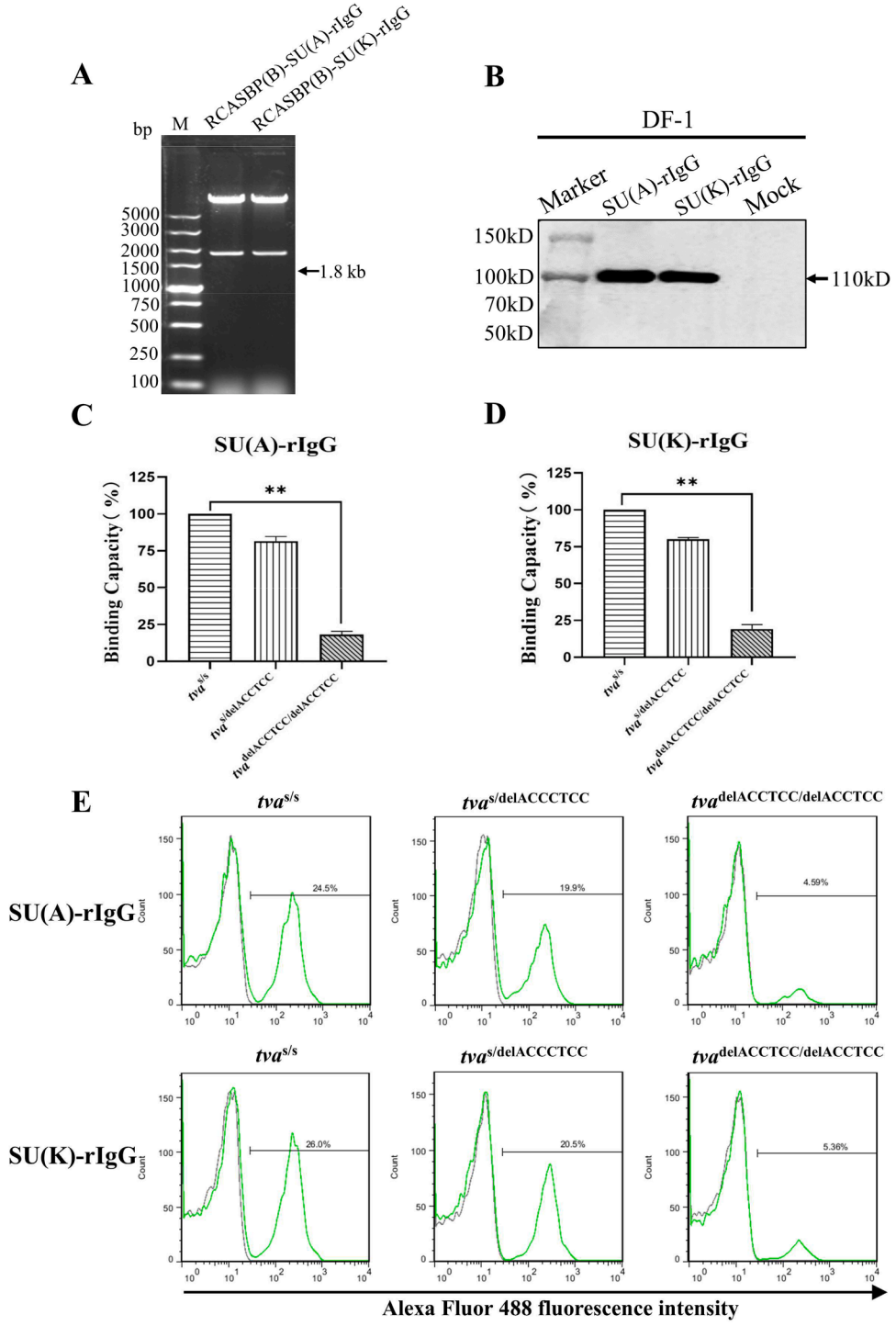

Fig. 4. Binding affinities of Tva receptor proteins ALV-A and ALV-K envelope glycoproteins. (A) Restriction enzyme digestion analysis of recombinant plasmids RCASBP(B)-SU(A)-rIgG and RCASBP(B)-SU(K)-rIgG. M: DNA Marker 5000, the sizes of diagnostic fragment which fused ALV SU gene and the Fc region of rabbit IgG are indicated on the left, and SU(A), and SU(K) represent the ALV-A, and ALV-K SU gene, respectively. **(B)** Western immunoblot analysis of the soluble forms of the SU glycoproteins SU(A)-rIgG and SU(K)-rIgG, immunoprecipitated with anti-rabbit IgG-agarose beads. Molecular masses (in kilodaltons) are given on the left. **(C and D)** The $tva^{s/s}$, $tva^{s/delACCTCC}$, and $tva^{delACCTCC/delACCTCC}$ CEFs were incubated with the same amount of SU(A)-rIgG or SU(K)-rIgG. The receptor-viral glycoprotein complexes were bound to goat anti-rabbit IgG linked to Alexa Fluor 488. The amount of Alexa Fluor bound to the cells was determined by FACS. The percentage of fluorescein-positive cells reflects the binding capacity of viral glycoproteins-Tva receptor protein. The binding capacity of SU(A)-rIgG or SU(K)-rIgG to Tva receptor protein expressed by the wild type $tva^{s/s}$ CEFs was set at 100%, and the binding capacity of SU(A)-rIgG or SU(K)-rIgG to Tva receptor protein expressed by $tva^{s/delACCTCC}$ and $tva^{delACCTCC/delACCTCC}$ genotypes CEFs was calculated according to its proportion. The values shown are averages and standard error from three experiments. To determine the statistical significance of differences between binding capacity of wild type and the mutant Tva receptor protein for SU(A)-rIgG or SU(K)-rIgG data sets, unpaired two-tailed *t* tests were performed (**, $P< 0.01$). **(E)** Representative examples of FACS histograms showing the percentages of Alexa Fluor 488-positive cells after incubation of $tva^{s/s}$, $tva^{s/delACCTCC}$, and $tva^{delACCTCC/delACCTCC}$ CEFs with the same amount of soluble proteins SU(A)-rIgG or SU(K)-rIgG. The fluorescein negative and positive cells are clearly different, as indicated by the presence of two separate peaks in the FACS histograms.

receptor is a crucial step that precedes virus entry into the host cell. In the context of ALV-A and ALV-K, the presence of T21 and S22 on the Tva receptor might provide the necessary structural and characteristics for a high-affinity interaction with the viral glycoproteins. Consequently, the absence of T21 and S22 due to the 6-nucleotide deletion is a reasonable explanation for the significant decrease in the ability of ALV-A and ALV-K to bind to and enter host cells. This proposed mechanism offers a plausible framework for understanding the phenotype of reduced ALV-A and ALV-K susceptibility associated with the *tva* receptor gene deletion, although further studies are still required to fully elucidate the precise molecular events involved.

In summary, our study identified two novel *tva* resistant alleles confer chickens resistance to infection by ALV-A and ALV-K due to 11-nucleotide (GCTGCCCACCC) insertion and 6-nucleotide (ACCTCC) deletion mutations in the *tva* receptor gene of Chinese Qingyuan partridge chicken lines. Our findings provides two valuable targets used for selective breeding of chickens toward the resistance to ALV-A and ALV-K, two important concerns of the poultry industry.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.104949.

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