Genetic mutations of avian leukosis virus subgroup J strains extended their host range

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The genetic diversity of avian leukosis virus subgroup J (ALV-J) is determined not only by the env gene, but also by its 3' UTR and 3' LTR. They all play important roles in extending the host range and tumour development. In the present study, one ALV-J strain (ZB110604-6) from Black-Bone Silky Fowl (BSF) and three ALV-J strains (ZB110604-3/4/5) from grey partridge (GP), which bore multiple tumours and breed in one house of Farm A, were demonstrated extending their host to GP, while two other ALV-J strains (LC110515-3/4) from BSF of Farm B could not infect the embryo fibroblast of GP. The BSF is a unique species of chicken in China, while the GP is a close relative of the pheasant that previously demonstrated resistance to ALV-J. Histopathology showed that various tumours were induced by ALV-J in the two species. Phylogenetic tree analysis showed that the isolates from Farms A and B, rather than species, belong to two different clusters of ALV-J. Genetic mutations analysis revealed that the isolates obtained from Farm A showed a higher frequency of mutation in the hypervariable region 2 domain than in other variable regions of the gp85 gene. From the nucleotide alignment of the 3' UTR and 3' LTR gene, and the spectrum of tumours observed in this study, we speculate that the deletions or mutations in the redundant transmembrane region, E element and U3 (CAAT boxes, CArG box and Y box) might associate with tumour formation and development. The extension of the host range of ALV-J to the GP suggested that housing different species together provides more opportunities for ALV-J to evolve rapidly.

INTRODUCTION

Avian leukosis virus subgroup J (ALV-J) is an oncogenic exogenous retrovirus of avians that was first isolated by Payne in meat-type chickens in the UK (Payne et al., 1991). Since then, it has spread worldwide and caused great losses in the poultry industry. Host-range studies showed that the domestic fowl, red jungle fowl, Sonnerat’s jungle fowl and turkey were susceptible to infection by ALV-J (HPRS-103), whereas the ring-necked pheasant, Japanese green pheasant, golden pheasant, Japanese quail, guinea-fowl, Peking duck, Muscovy duck and goose were resistant (Payne et al., 1992b). Clinical infection is associated with immune tolerance, high mortality, delayed growth and the development of a variety of tumours, including myelocytomas, erythroblastomas, haemangioiomas, nephromas and sarcomas (Arshad et al., 1997a; Fadly & Payne, 2003; Landman et al., 2002; Payne, 1998; Payne et al., 1992a; Sironi et al., 2006; Stedman & Brown, 1999, 2002). Myelocytomatosis is the most commonly reported neoplastic disease induced by ALV-J in meat-type chickens (Arshad et al., 1997b; Chesters et al., 2001; Nakamura et al., 2000; Payne, 1998; Stedman & Brown, 1999). No neoplastic diseases induced by ALV-J were found in layer chickens until 2002. In 2002, myelocytomatosis was observed in some commercial White Leghorn egg-layer flocks, and a recombinant avian leukosis virus (ALV) containing the LTR of ALV subgroup J and the envelope of ALV subgroup B was isolated (Gingerich et al., 2002). In 2004, the first field case of myeloid leukosis caused by ALV-J in commercial egg-type chickens was reported in China (Xu et al., 2004). The ADOL-Hcl strain of ALV-J was used to induce lymphoid leukosis and haemangioiomas experimentally in White Leghorn chickens (Williams et al., 2004). From 2007 to 2011, haemangioiomas associated with ALV-J broke out in layer flocks in China (Cheng et al., 2010; Gao et al., 2012; Lai et al., 2011; Shi et al., 2011; Wu et al., 2010).

Genetic and antigenic variations with sequence changes in the variable regions of the env gene were identified in all of
the isolates obtained from meat-type and egg-type chickens. The genetic diversity of ALV-J is determined not only by the gp85 env gene, but also its 3’ UTR (Zavala et al., 2007). They both play important roles in tumour development and extending the host range. In fact, the host range of ALV is determined by the two host-range-determining regions of the gp85 env gene, hypervariable regions 1 and 2 (hr1 and hr2) (Rainey et al., 2003).

In the present study, we demonstrate that four strains of ALV-J isolated from a mix breeding of Black-Bone Silky Fowl (BSF) and grey partridge (GP) extended their host to GP, a resistant species of ALV-J previously. The BSF, a unique breed of chicken native to South China, is a bird with snow-white silky feathers and black-coloured bones, meat and skin (Tian et al., 2007). The GP is a close relative of pheasant and grouse, and are very popular game birds (Tapper et al., 1996). The pheasant family has been demonstrated to be resistant to ALV-J (Payne et al., 1992b). The spectrum of ALV-J-induced tumours and genetic mutations of ALV-J isolates are described in the paper.

RESULTS

Gross and histopathology

The spectrum of tumours induced by each ALV-J isolate was identified by microscopy. The results are shown in Table 1. Myelocytoma (5/6) was the predominant type of tumour induced by ALV-J in the three flocks. Other types of tumours, including lymphocytoma (4/6), cholangiocarcinoma (3/6), haemangioma (2/6), erythroblastosis (2/6), fibrosarcoma (2/6) and aneurysm (1/6), were sometimes found in birds that had myelocytomas. The gross and histopathological morphology of the myelocytomas, fibrosarcomas and lymphosarcomas were similar to those previously reported (Cheng et al., 2010). The haemangiomas look like bright-red strawberries or peanuts and were found on the claws (Fig. 1a), the lungs, pancreas, intestines or the heart. Haemangiomas can be classified into two types by histology, the phlebangioma (claw and lung) (Fig. 1b) and the aneurysm (pancreas, intestine and heart) (Fig. 1c). Phlebangioma are unencapsulated aggregates of closely packed, thin-walled capillaries, usually with an endothelial lining. The blood-filled vessels are separated by scant connective tissue. Their lumens may be thrombosed or organized. Aneurysms are spherical in shape and involve only a portion of the vessel wall when they occur in small arteries. They are often filled, either partially or fully, by a thrombus. Erythroblastosis was a common lesion in the ALV-J-infected birds in this study. Most of the normal erythrocytes in the peripheral blood and visceral tissues were replaced by erythroblasts. The erythroblasts were polymorphic (spherical and elliptical) and were larger, with chromatin packed less densely compared with normal erythrocytes. Their cytoplasm was basophilic and contained vacuoles surrounding the spherical or elliptical nuclei (Fig. 1d). One of the rare ALV-induced tumours, a cholangiocarcinoma, was first found in BSFs infected by ALV-J isolates. A cholangiocarcinoma is a primary malignant tumour originating from cells that resemble biliary epithelium. Their gross appearance is that of one or more small, firm, white masses in the intrahepatic bile ducts. Microscopically, cholangiocarcinoma are found to comprise many crowded mutated epithelial cells that originated in the bile ducts (Fig. 1e).

Virus isolation and identification

The ELISA results demonstrated that the isolates were positive group-specific antigen (p27); PCR products were obtained using the primers specific for ALV-J and not with the primers for Marek’s disease virus (MDV); the immunofluorescence assay (IFA) results showed that the isolates reacted with the ALV-J antibody and did not react with the reticuloendotheliosis virus (REV) antibody (Fig. 2). These data demonstrate that the tumours found in the three flocks were induced by ALV-J. A total of six strains of ALV-J were isolated. Their PCR products were successfully cloned and sequenced. The proviral genes of the six isolates were determined after assembling contiguous sequences for each of the viruses. The GenBank accession numbers are listed in Table 1. For verifying the host of ALV-J isolates extending to GP, embryo fibroblasts from GP were used to be infected by all the isolates. The results of IFA and p27 show that the strains (ZB110604-3/4/5/6) from Farm A (mix breeding BSF and GP in one house) did infect embryo fibroblasts of GP other than BSF strains (Fig. 3; p27 result not shown).

Genetic variations in the env gene of the ALV-J isolates

The nucleotide changes in the gp85 env gene of the six isolates showed a maximal divergence of 14.6 %, with nucleotide sequence identities ranging from 86.3 % to 94.6 %. Analysis of the deduced amino acid sequences revealed that the maximal divergence in the amino acid sequence was 8.9 %, with sequence identities ranging from 91.7 % to 95.8 % (Fig. 4), suggesting that they originated from ALV-J. The phylogenetic analysis indicated that the six isolates of ALV-J belong to two clusters (Fig. 5). The LC110515-3 and LC110515-4 strains from the BSF showed close homology with the Chinese isolate JS09Gy6 (98.4 % and 95.4 %, respectively) that cause haemangiomas in layer chickens. The strains of ZB110604-3/4/5 (from the GP) and ZB110604-6 (from the BSF) showed relatively independent cluster between the prototype UK strain HPRS-103 and US strain ADOL-7501. These data indicate that the strains from Farm A and Farm B originated from different sources.

The amino acid alignments showed that genetic mutations of all six of the isolates were distributed throughout the envelope surface (SU) glycoprotein (Fig. 6). In four of the isolates from Farm A, significant mutations and deletions were present mainly in amino acids 46–68, the hr1 and hr2
variable domains of the gp85 gene, and particularly in hr2 in three of the isolates from the GP and one isolate from the BSF. The hr2 domain is most likely the main determining factor for the extension of the host range of ALV-J to include the GP.

The gp37 sequences were relatively conserved. The extreme amino end of gp37 contains the only hypervariable region. Two amino acid deletions had occurred in the hypervariable region of ZB110604-4. There was a substitution of Ile89 in heptad repeat 1 (HR1) of ZB110604-3/4/5, whereas heptad repeat 2 (HR2) (amino acids 72–110) of all six of the isolates was more highly conserved (data not shown).

Molecular characterization of 3' UTR of ALV-J strains

The comparison of the nucleotide sequences of the 3' UTR is shown in Fig. 7. A total of 120 bp from the 5' end and 2 bp from the 3' end of the non-functional redundant transmembrane region (rTM) were deleted in all six of the isolates. The direct repeat element (DR-1) is present in all six of the isolates, and 1 bp in LC110515-3/4, 6 bp in ZB110604-3/4/5 and 7 bp in ZB110604-6 were deleted from the 5' end of DR-1. A complete E element is present in the LC110515-3/4, ZB110604-4 and ZB110604-6 strains, and two fragments containing 95 bp and 33 bp were deleted from the E element in the ZB110604-3/4 strains.

Using the consensus sequence of U3 for the isolates studied, the typical retroviral transcriptional regulatory elements, the CAAT boxes, CArG box and Y box motifs, contained variations, whereas the PRE box, TATA box and polyA were fairly well conserved (Fig. 4). In the ZB110604-6 strain, the fifth nucleotide (G) of the CAAT box located at position 10-18 is replaced with an adenine; in the LC110515-3 strain, the third nucleotide (T) of the 5' CArG box located at position 50-52 is replaced with an adenine;

Table 1. Accession number of ALV-J strains in GenBank and neoplastic spectrum in two species

<table>
<thead>
<tr>
<th>Farm</th>
<th>Species</th>
<th>Strain name</th>
<th>Accession number</th>
<th>Neoplastic spectrum*</th>
</tr>
</thead>
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<tr>
<td>A</td>
<td>GP</td>
<td>ZB110604-3</td>
<td>KC841154</td>
<td>ML (liver, spleen), LL (liver), FS (pectoralis)</td>
</tr>
<tr>
<td></td>
<td>GP</td>
<td>ZB110604-4</td>
<td>KC841155</td>
<td>ML (liver, BM), LL (liver, BM), HG (lung)</td>
</tr>
<tr>
<td></td>
<td>GP</td>
<td>ZB110604-5</td>
<td>KC841156</td>
<td>ML (liver), EB (liver, kidney)</td>
</tr>
<tr>
<td></td>
<td>BSF</td>
<td>ZB110604-6</td>
<td>KC841157</td>
<td>ML (liver, spleen, intestine), LL (liver, spleen, lung), CC (liver), AN (pancreas, intestine), FS (pectoralis)</td>
</tr>
<tr>
<td>B</td>
<td>BSF</td>
<td>LC110515-3</td>
<td>KC841152</td>
<td>ML (liver, spleen, lung, kidney), HG (claw), CC (liver), EB (all tissues)</td>
</tr>
<tr>
<td></td>
<td>BSF</td>
<td>LC110515-4</td>
<td>KC841153</td>
<td>LL (liver, lung), CC (liver)</td>
</tr>
</tbody>
</table>

*ML, Myelocytomas; HG, haemangiomas; CC, cholangiocarcinoma; EB, erythroblastosis; LL, lymphocytomas; FS, fibrosarcoma; AN, aneurysm; BM, bone marrow.

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Fig. 1. Multiple tumours were induced by the ALV-J isolates. (a) Haemangiomas on the claw of a BSF; (b) phlebangioma; (c) aneurysm; (d) erythroblastosis; (e) histology of a cholangiocarcinoma.
DISCUSSION

The envelope protein of ALV is generally cleaved into two functionally distinct domains. The N-terminal subunit (SU glycoprotein, encoded by the gp85 gene) usually contains all of the receptor-binding capability and is therefore an important determinant of host range. The C-terminal subunit (TM glycoprotein, encoded by the gp37 gene) is the membrane-anchored subunit, and it includes elements critical for mediating membrane fusion (Netter et al., 2004). The gp85 genes in the ALV-J isolates exhibited remarkable variability within very similar regions (vr2, hr1, hr2 and vr3) (Silva et al., 2000). The pattern of variability suggests that this region was able to evolve very rapidly to produce the isolated strains, suggesting that this ability allows adaptation to a variety of environmental pressures in nature (Thu & Wang, 2003). Fast genetic shift and drift may generate new phenotypes such as a different host range and antigenicity.

In the present paper, we have shown that ALV-J was transmitted to a new host species, the GP, which belongs to the pheasant family that previously showed resistance to ALV-J, and infected a unique species of chicken, the BSF. Multiple tumours were observed in sick birds, including myelocytomas, lymphocytomas, cholangiocarcinoma, haemangioas, erythroblastos and fibrosarcomas. Among these tumours, cholangiocarcinoma induced by ALV-J was found for the first time. Cholangiocarcinoma is a form of cancer that is composed of mutated epithelial cells that originate in the bile ducts which drain bile from the liver into the small intestine. Cholangiocarcinoma is a relatively rare neoplasm that is classified as an adenocarcinoma. In humans, a number of risk factors for the development of cholangiocarcinoma have been described, including primary sclerosing cholangitis, ulcerative colitis, parasitic liver diseases and viral pathogens, such as hepatitis B or hepatitis C virus and human immunodeficiency virus (HIV) (Bergquist et al., 2002; Chapman, 1999; Shin et al., 1996; Watanapa & Watanapa, 2002). HIV and ALV-J are both classified as retrovirus and they might induce cholangiocarcinoma through similar pathogenic mechanisms, although it is unclear whether the virus itself or other correlated and confounding factors caused this tumour.

The phylogenetic tree analysis revealed that two of the ALV-J isolates from the BSF from Farm B originated from layer strains (JS09GY6), while four of the isolates from GP and BSF from Farm A belonged to an independent cluster. The genetic mutation analysis revealed that the ALV-J isolates from Farm A had more mutations in the hr2 domain of the gp85 gene. Therefore, we speculate that the hr2 domain is more important than the hr1 domain in host-range determination. Lupiani had reported that not only the envelope gene, but also other elements of the viral genome play an important role in the pathogenesis of ALV.
Lupiani et al., 2003). By comparing the amino acid alignment with the spectrum of tumors discovered in this study, we found that deletions or mutations in the rTM, E element and U3 (CAAT boxes, CArG box and Y box) did not affect the formation of myelocytomas. These regions may be associated with the development of other types of tumors.

Interesting, although the ZB110604-6 strain was isolated from the BSF, it exhibited different molecular characteristics and pathogenicity (tumor spectrum) from those of the isolates from the same species on Farm B. These results indicate that housing more than one species together provides opportunities for the rapid evolution of ALV-J.

**Fig. 4.** Alignment of the amino acids in the gp85 glycoprotein of the ALV-J isolates. Mutations and deletions present mainly in hr2 of four isolates from Farm A. ZB110604-3/4/5 is the sequence obtained from the GP from Farm A. ZB110604-6 is the sequence obtained from the BSF from Farm A. LC represents the sequence amplified from the BSF from Farm B. Dots indicate amino acids that are identical with each other. Dashes indicate gaps that were introduced to improve the alignment.

**Fig. 5.** Phylogenetic tree of gp85 glycoproteins. The tree shows that the six isolates belong to two clusters. A CLUSTAL W alignment constructed by the 5.01 version of the MEGALIGN function in the DNASTAR DNA analysis software was used.
sections were stained with haematoxylin and eosin for observation. In 2011, 12 sick birds were sent to our lab for diagnosis. The basic information about them is shown in Table 2. Based on these data, housing different species of birds in the same place and with each other is strongly recommended.

### METHODS

#### Case history
In 2011, 12 sick birds were sent to our lab for diagnosis. The basic information about them is shown in Table 2. Viral culture and antigen assay. Serum and the liver, spleen, intestine, muscle, ovary, brain, bone marrow and discrete nodules of liver, spleen, heart, lung, kidney, proventriculus, ventriculus, tumours in various visceral organs. The farmers doubted that the birds had different origins. The birds from the three flocks were bred in different farms is approximately 400 km. The day-old birds from the two farms and one flock of the three flocks were bred in the same place and separated by a net. The birds from the three flocks were bred in three-storey cages and received routine layer feed, municipal drinking water and vaccinations. The first clinical signs of disease that the farmers noticed were decreasing egg production and haemangiomatis on the skin. Approximately 30–40 % of the birds in these farms were taken out of production. The affected birds exhibited wasting, paralysis, abdominal distension, diarrhoea and anaemia. At autopsy, the birds selected because they were clinically ill or had died had tumours in various visceral organs. The farmers doubted that the birds were infected with ALV-J.

#### Gross and histopathology
The birds were euthanized by cervical dislocation and necropsied. The gross lesions were examined. Samples of liver, spleen, heart, lung, kidney, proventriculus, ventriculus, intestine, muscle, ovary, brain, bone marrow and discrete nodules were removed from the birds and fixed in 10% neutral buffered formalin. The tissues were processed for standard paraffin embedding, sections of approximately 4 μm thickness were obtained and the sections were stained with haematoxylin and eosin for observation.

#### Virus culture and antigen assay
Serum and liver, spleen, kidney and tumours of each bird were collected and stored at –80 °C. Filtered sera or homogenates of the tissues were inoculated in DF-1 cell (or primary chicken embryo fibroblasts of 9 day-old embryos from GP) cultures [Dubbeco’s modified Eagle’s medium (Invitrogen)] and then incubated at 37 °C with two serial passages performed at 7 day intervals. Subsequently, cell lysates were prepared by three cycles of freeze-thaw and they were tested for ALV group-specific antigen (gsa) (p27) by an ELISA using anti-p27 antibody-coated plates (IDEXX Laboratories). The IFA was performed on DF-1 cells using subgroup J or reticuloendotheliosis virus (REV)-specific antibodies on either unixed or acetone–ethanol (3:2) fixed cells. The infected DF-1 cells were initially stained with either ALV-J-specific polyclonal rabbit serum or REV mAb (prepared in our lab). After the unbound primary antibodies were washed away with PBS, the cells were stained with FITC-labelled anti-rabbit or anti-mouse immunoglobulins (Sigma) mounted in buffered glycerol (50%) and observed with a fluorescence microscope.

#### PCR, cloning and sequencing
Proviral genomic DNA from the DF-1 cultures was extracted and the viral genes were identified by PCR. Normal specific pathogen-free chicken tissue cultures and the NX0101 strain of ALV-J and the vaccine strain of MDV were used as the negative and positive controls, respectively. These controls were incubated and processed under the same conditions as were the samples. The primers designed specifically for ALV-J were as follows: forward primer, 5′-GGACATCACCCCAAGGATGA-3′; reverse primer, 5′-GTCAGGGAATCGACGGTC-3′. This primer set covered part of the pol gene, all of the env gene, and the 3′ UTR and 3′ LTR. The primers designed specifically for MDV were as follows: forward primer, 5′-TACTTCTTATATAGATTGAGACGT-3′; reverse primer, 5′-GAGATCCCTGTAAGGTGTAATA-3′. The amplified genes were purified using an agarose gel DNA extraction kit (Takara) and cloned into the pMD18-T vector according to the manufacturer’s instructions. The recombinant plasmids were transformed into DH5α competent cells. Single colonies were selected and cultured for plasmid extraction. The DNA sequences of the inserts in the positive clones were determined by the Biotechnology Company. To avoid PCR errors, the DNA sequencing was performed twice independently.

#### Multiple alignments and phylogenetic analysis
Nucleotide and amino acid sequence analyses were performed using the amplified gene. The generated consensus sequence comprised sequences of the isolates obtained in this study and reference sequences. Multiple alignments were accomplished using the CLUSTAL W method (MEGALIGN sequence analysis software, DNASTAR version 4.03; DNASTAR). A phylogenetic analysis of the sequences was accomplished using a CLUSTAL W alignment constructed by the 5.01 version of the MEGALIGN function in the DNASTAR DNA analysis software (DNASTAR). GenBank accession numbers for the control reference sequences used in the phylogenetic analysis reported in this study are: ADOL-7501, JS09 Gy6(2010).seq, SD09 DP03(2011).seq, J1L10 HW02(2012).seq, LC110515-3(BSF).seq, LC110515-4(BSF).seq, ZB110604-6(BSF).seq, ZB110604-3(GP).seq, ZB110604-4(GP).seq, ZB110604-5(GP).seq, and ADOL-7501.seq.
**Fig. 7.** Transcriptional regulatory elements in the 3' UTR of ALV-J isolates from the BSF and the GP.
**Table 2. Basic information of infected flocks**

<table>
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<tr>
<th>Farm</th>
<th>Source</th>
<th>Species</th>
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<th>Age (day)</th>
<th>Mortality (%)</th>
<th>Number of cases</th>
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<td>ZB</td>
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<tr>
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<td>5</td>
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</table>

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