Prevalence of Fowl Glioma-Inducing Virus in Chickens of Zoological Gardens in Japan and Nucleotide Variation in the env Gene

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ABSTRACT. Fowl glioma-inducing virus (FGV), which belongs to subgroup A of avian leucosis virus (ALV), is tumorigenic in the nervous system. In a zoological garden in Japan, approximately 40% of chickens, including Japanese fowls, were infected with FGV. Because this zoological garden plays a role as a major supplier of Japanese fowls for other zoological gardens, FGV infection is suspected to have spread among ornamental chickens. In this study, the prevalence of the disease was examined in a total of 129 chickens in three other zoological gardens by nested polymerase chain reaction (PCR), reverse transcription nested PCR and enzyme-linked immunosorbent assay. Twenty-six to 56 percent of the fowls in each of the examined gardens were positive by nested PCR. The phylogenetic analysis revealed that the 3' untranslated region, including the specific sequence of FGV, of the 14 isolated ALVs showed high sequence identity and a close relationship with FGV. In addition, the env gene of the isolates frequently showed mutations and deletions of nucleotides. These results suggest that FGV is prevalent among ornamental chickens kept in zoological gardens in Japan.

KEY WORDS: avian leucosis virus, fowl glioma, gene mutation, prevalence.

Avian leukemia viruses (ALVs) are the most naturally occurring avian retroviruses associated with various neoplasms as well as lymphoid leukemia. The env gene of ALV genome codes for the following 2 proteins: surface component (SU, gp85) and transmembrane component (gp37). ALVs occurring in chickens are classified into six subgroups, A, B, C, D, E and J, on the basis of the differences in SU [8]. The mechanism of tumorigenesis by ALVs is interpreted as follows: the ALV genome integrate upstream of a host proto- oncogene as a provirus and induces tumors by increasing the expression of the proto-oncogene expression via the promoter and enhancer functions of the long terminal repeats [18]. We have recently demonstrated that so-called fowl glioma is caused by fowl glioma-inducing virus (FGV), which is a strain of subgroup A of ALV [10, 15, 23], and have already determined the complete nucleotide sequence of FGV [24]. So-called fowl glioma had been a poorly understood entity characterized by multiple nodular gliomatous growths associated with disseminated nonsuppurative encephalitis [20, 21]. This disease has been previously described as glioma [1, 12], astrocytoma [14, 17], astroblastoma, multiple glioblastoma, mixed gliomatosis [2], epizootic gliosis and astrocytoma [26].

ALV is spread by vertical and horizontal transmission [8]. At the present time, there is no effective treatment for this viral infection. The best way to control the avian disease is the elimination of vertical shedders from breeding flocks containing infected birds. However, this measure is not realistic for Japanese fowls for the following reasons. Japanese fowls are designated as natural treasures in Japan. The appearance of each breed of Japanese fowl has been strictly determined, and it is difficult and laborious to preserve the breeds. In fact, the number of Japanese fowl is decreasing in this country each year. To eradicate ALV infection in commercial chickens, breeder hens are tested for the virus and those that test positive are discarded [8]. Therefore, to control FGV infection in fowl, the detection and removal of infected birds is appropriate. We previously performed an etiological study in 131 Japanese fowls kept in zoological garden A, which is a major supplier of fowl for other domestic zoological gardens, and reported that 39.7% of these birds were positive for FGV proviral DNA by polymerase chain reaction (PCR) [9]. Based on this report, FGV was suspected to have spread in the flocks of chickens in several zoological gardens in Japan.

In the present study, we elucidated the prevalence rate of FGV among chickens in three other zoological gardens in Japan using the previously established PCR-based assay [9] and analyzed the env gene and 3' untranslated region (3' UTR), which is located upstream of the 3' long terminal repeat, of isolated viruses to clarify the molecular phylogenesis of FGV and the isolated viruses.

MATERIALS AND METHODS

Prevalence of FGV in three flocks: Japanese fowls and fertile eggs in zoological garden A in Toyama prefecture were transported to 10 zoological gardens in Hokkaido, Saitama, Tokyo, Kanagawa, Ishikawa, Aichi and Kyoto. We examined 129 chickens in three zoological gardens (B
in Hokkaido, C and D in Tokyo), which had obtained the fowl from zoological garden A. The number of examined birds was 69, including Japanese bantam, Silky, Australorp and Rhode Island red, in the zoological garden B, 36 birds, including Japanese bantam, Silky and Nagoya in zoological garden C, and 24 Japanese bantam in zoological garden D. They were approximately 1.4 to 3 years of age. The presence of ALVs in the cloacal swab was examined by enzyme-linked immunosorbent assay (ELISA), and the 3′ UTR of the FGV genome was detected by nested PCR and reverse transcription (RT)-nested PCR. When the provirus of FGV is detected from feather pulp, it is already disseminated in many organs [9]. Nucleic acids for the nested PCR and RT-nested PCR were extracted from the feather pulp of live birds. All PCR tests were performed in duplicate. Seventeen birds in zoological garden B and three in garden D were necropsied after their death and the brains were collected from the birds for detection of the viral genome by nested PCR and histopathology.

**DNA extraction:** Total DNA samples were prepared from the feather pulp or brain tissues of all examined birds by standard phenol-chloroform extraction and ethanol precipitation.

**RNA extraction and cDNA synthesis:** Total RNA was extracted from feather pulp using the TRIzol reagent (Invitrogen, California, U.S.A.) according to the manufacturer’s instructions. The extracted RNA was treated with DNA-free (Ambion, Texas, U.S.A.) following the manufacturer’s instructions. RT was performed using random hexamers (Takara Bio, Shiga, Japan) and SuperScript II RNase H-Reverse Transcriptase (Invitrogen) following the manufacturer’s instructions. As an internal control for RNA extraction and cDNA synthesis, the PCR amplification was performed for all cDNA samples using primers specific to chicken β-actin (Table 1). The PCR amplification for chicken β-actin was performed following condition: denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec.

**Oligonucleotide primers:** The oligonucleotide primers for the detection of FGV were designed based on the 3′ UTR of the viral genome (Table 1; GenBank accession No. AB112960). A 135-bp fragment was amplified as a target sequence using internal primers 5FGOG and 3FGOG. For sequencing of the env gene and a specific region in the 3′ UTR, we used primers H5 and L7Krev. H5 was designed by Hatai et al. [9] based on the ALV HPRS103 sequence (GenBank accession No. Z46390).

**Nested PCR:** For the detection of FGV, specific nested PCR was performed by the modified procedure established by Hatai et al. [9]. The PCR was performed using Takara Ex Taq HS (Takara Bio) and genomic DNA or undiluted cDNA. In the second round, undiluted first-round PCR product was added to reaction mixture. The first round PCR amplifications were performed using the following profile: 98°C for 1 min, followed by 25 cycles of 98°C for 10 sec, 60°C for 15 sec, and 72°C for 30 sec using GeneAmp PCR System 9700 (Applied Biosystems) with Max ramp speed mode. In the second round, the reaction cycles and the extension time were changed to 30 cycles and 15 sec, respectively. The PCR products were analyzed by 2.5% agarose gel electrophoresis.

**ELISA for detection of ALV antigens:** Supernatants of cloacal swab suspensions obtained from birds were examined for ALV antigen using the ELISA kit ‘FlockChek’ (IDEXX, Maine, U.S.A.) detecting group-specific antigen p27. The supernatants were prepared according to the method previously described [7].

**Histopathology:** The brains were fixed in 20% neutral buffered formalin, routinely processed, and embedded in paraffin wax. Sections (4 μm) were cut and stained with hematoxylin and eosin (HE) and examined by light microscopy.

**Cell culture and virus isolation:** DF-1 cells were obtained from ATCC (Manassas, Virginia, U.S.A.) and cultured in Dulbecco’s modified Eagle medium supplemented with L-glutamine, glucose and fetal bovine serum. DF-1 cells in growth medium were plated in a 25-cm² plastic flask (Becton Dickinson, Franklin Lake, New Jersey, U.S.A.) and inoculated with 0.5 ml of supernatants of cloacal swab suspensions which were obtained from fowls positive for nested PCR and ELISA. Culture supernatants and cells were harvested 7 days after inoculation. Total RNA was extracted from harvested cells and cDNA was synthesized for sequencing following the above method.

**Sequencing and phylogenetic analysis:** For sequencing the env gene and the FGV-specific sequence in the 3′ UTR, PCR was performed using primer pair H5 and L7Krev (Table 1). The PCR was performed using PrimeSTAR HS DNA Polymerase (Takara Bio) and the following profile: 98°C for 1 min, followed by 30 cycles of 98°C for 10 sec,

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Primer position</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>EnvFw3a</td>
<td>5′-ACGTTGGCGGGAATGTGTGT-3′</td>
<td>6558–6577</td>
<td>AB112960</td>
</tr>
<tr>
<td>L7Krevb</td>
<td>5′-CTATACACACCTAGTCCC-3′</td>
<td>7105–7123</td>
<td>AB112960</td>
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<tr>
<td>5FGGB</td>
<td>5′-TGTAGGCGGGCTCTTGTATT-3′</td>
<td>6894–6913</td>
<td>AB112960</td>
</tr>
<tr>
<td>3FGGC</td>
<td>5′-TTGTCGGTCAAGCCTTGCCTT-3′</td>
<td>7008–7028</td>
<td>AB112960</td>
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<tr>
<td>β-actin 5′</td>
<td>5′-TATCCGAAGATCTGTATG-3′</td>
<td>932–951</td>
<td>L08165</td>
</tr>
<tr>
<td>β-actin 3′</td>
<td>5′-ATCTCGTCTTGTTTTATGCG-3′</td>
<td>1250–1269</td>
<td>L08165</td>
</tr>
<tr>
<td>H5</td>
<td>5′-GGATGAGGTGACTAAGAAAG-3′</td>
<td>5258–5277</td>
<td>AB112960</td>
</tr>
</tbody>
</table>

a) External primers for nested PCR. b) Internal primers for nested PCR.

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Table 1. Sequences of oligonucleotide primers and targets
Prevalence of FGV: The screening for FGV infection was performed in the flocks of three zoological gardens, B, C, and D, in Japan. These birds were divided into six groups based on the results of nested PCR, RT-nested PCR and ELISA (Table 2). Some chickens, including 20.3% of the examined birds in B, 50% in C and 20.8% in D, showed positive results in all three tests and they were classified into group 1. The birds in group 2, including 2.9% in B and 8.3% in D, were positive by nested PCR and RT-nested PCR but negative for the ALV antigen p27 in cloacal swab by ELISA. Group 3, including 2.9% in B, 2.8% in C and 4.2% in D, was positive by nested PCR and ELISA but negative by RT-nested PCR. One bird (2.8%) in C was positive only by nested PCR and was classified into group 4. The birds in group 5, including 56.5% in B, 30.6% in C and 45.8% in D, were positive just by ELISA. In group 6, including 17.4% of the birds in B, 13.9% in C and 20.8% in D, the birds were negative for nested PCR and ELISA.

Histopathology of brains in nested PCR-positive chickens: Birds that died after the examination of the prevalence were necropsied. All necropsied birds had no gross lesions. The brains were obtained from 4 chickens in zoological garden A (Tym_26, Tym_34, Tym_43 and Tym_73), one chicken in zoological garden B (Sp_49), one chicken in zoological garden C (Ed_2) and 5 chickens in zoological garden D (U_1, U_3, U_14, U_17, and U_22). The identities of these 11 isolates with FGV ranged only from 51 to 53% due to some deletions and frequent nucleotide substitutions. Other isolates were obtained from 4 chickens in zoological garden A (Tym_26, Tym_34, Tym_43 and Tym_73), one chicken in zoological garden B (Sp_49), one chicken in zoological garden C (Ed_2) and 5 chickens in zoological garden D (U_1, U_3, U_14, U_17, and U_22). The identities of these 11 isolates with FGV ranged from 92 to 93%. The env gene of the isolates showed 88 to 95% nucleotide identity to the corresponding sequence of FGV. In the variable region in the env gene (sequence position in FGV: 5649–5932), the identities of Sp_40, Sp_50 and Sp_53 isolated from chickens in zoological garden B with FGV ranged only from 51 to 53% due to some deletions and frequent nucleotide substitutions. Other isolates were obtained from 4 chickens in zoological garden A (Tym_26, Tym_34, Tym_43 and Tym_73), one chicken in zoological garden B (Sp_49), one chicken in zoological garden C (Ed_2) and 5 chickens in zoological garden D (U_1, U_3, U_14, U_17, and U_22). The identities of these 11 isolates with FGV ranged from 93 to 96%. The nucleotide sequences of the isolated viruses were analyzed for their phylogenetic relationship with seven ALSVs and FGV based on the env gene (Fig. 2). Eleven viruses were categorized into the same group as FGV, whereas Sp_40, Sp_50 and Sp_53, were categorized into another group.

DISCUSSION

In this study, we have shown the prevalence (26–56%) of FGV infection in chickens of 3 zoological gardens using ELISA and the PCR-based assays we previously established [9]. The viruses isolated from PCR-positive chickens had

Table 2. Results of nested PCR, RT-nested PCR and ELISA in chickens in three zoological gardens

<table>
<thead>
<tr>
<th>Group</th>
<th>Nested PCR</th>
<th>RT-nested PCR</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>C</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>–</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>–</td>
<td>ND</td>
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</tr>
<tr>
<td>Total</td>
<td></td>
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<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nested PCR</td>
<td>14 (20.3)</td>
<td>18 (50)</td>
<td>5 (20.8)</td>
</tr>
<tr>
<td>RT-nested PCR</td>
<td>2 (2.9)</td>
<td>0 (0)</td>
<td>2 (8.3)</td>
</tr>
<tr>
<td>ELISA</td>
<td>2 (2.9)</td>
<td>1 (2.8)</td>
<td>1 (4.2)</td>
</tr>
<tr>
<td></td>
<td>0 (0)</td>
<td>1 (2.8)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>39 (56.5)</td>
<td>11 (30.6)</td>
<td>11 (45.8)</td>
</tr>
<tr>
<td></td>
<td>12 (17.4)</td>
<td>5 (13.9)</td>
<td>5 (20.8)</td>
</tr>
</tbody>
</table>

a) Not done. b) The number of categorized birds. The percentage is given in parentheses.

56°C for 5 sec, and 72°C for 2 min using GeneAmp PCR System 9700 (Applied Biosystems) with Max ramp speed mode. After agarose gel electrophoresis, single-band PCR products were subcloned. Subcloned plasmid DNA was purified using the QIAprep Spin Miniprep kit (QIAGEN). Sequencing was performed by Bio Matrix Research (Chiba, Japan). The phylogenetic analysis were performed along with bootstrap analysis using CLUSTAL W 1.8.3 [22], and the BLAST program (National Center for Biotechnology Information) search of GenBank was performed to check for homology with known avian leukosis/sarcoma viruses (ALSVs) gene sequences. The NJplot program [16] was used to draw the graphical output of the phylogenetic tree generated by the neighbor-joining method.

RESULTS

Sequence and phylogenetic analysis: Sequence analysis of an FGV-specific sequence in the 3' UTR and of the env gene was performed for isolated viruses that were obtained from the 14 birds belonging to group 1 in Table 2. The sequence identity of the specific sequence in the 3' UTR (sequence position in FGV: 6894–7028, GenBank accession No. AB112690) between FGV and the 14 isolates ranged from 92 to 93%. The env gene of the isolates showed 88 to 95% nucleotide identity to the corresponding sequence of FGV. In the variable region in the env gene (sequence position in FGV: 5649–5932), the identities of Sp_40, Sp_50 and Sp_53 isolated from chickens in zoological garden B with FGV ranged only from 51 to 53% due to some deletions and frequent nucleotide substitutions. Other isolates were obtained from 4 chickens in zoological garden A (Tym_26, Tym_34, Tym_43 and Tym_73), one chicken in zoological garden B (Sp_49), one chicken in zoological garden C (Ed_2) and 5 chickens in zoological garden D (U_1, U_3, U_14, U_17, and U_22). The identities of these 11 isolates with FGV ranged from 93 to 96%. The nucleotide sequences of the isolated viruses were analyzed for their phylogenetic relationship with seven ALSVs and FGV based on the env gene (Fig. 2). Eleven viruses were categorized into the same group as FGV, whereas Sp_40, Sp_50 and Sp_53, were categorized into another group.
only minute mutations in the specific sequence in 3' UTR detected by nested PCR, but the env gene showed 51–96% homology between the isolates and FGV, suggesting presence of nucleotide variations of the env gene of FGV.

As we have previously discussed the results of the three assays [9], group 1 in Table 2 had a perfect correlation using the three assays. Groups 1–4 were positive by nested PCR. The positive rate for nested PCR in the examined zoological gardens ranged from 26.1% to 55.6%. Group 2 was considered to have shed no viral antigen. Because detection of the proviral DNA is generally more sensitive than detection of the viral RNA, the results of group 3 appear to be reflected by the fact. One bird belonging to group 4 was positive by nested PCR, but negative by RT-nested PCR and ELISA. It was considered that the proviral DNA of FGV had been integrated into the host genome but RNA transcription had not been promoted at the time of sample collection in this bird. Although the birds positive by ELISA in group 5 were considered to shed the group specific antigen of other ALVs, including endogenous retroviruses, into the cloaca [5], the birds in groups 1 and 3 might have shed the antigen of FGV and/or other ALVs. To eradicate FGV and ALV contamination from chicken flocks in these zoological gardens, it is recommended that chickens in group 6 are separated from chickens classified into other groups and that only they are used for future breeding.

A 135-bp sequence in the 3' UTR of FGV was used as the target of nested PCR for detection of FGV [9] because the region has low similarity to other ALSVs and is considered to be a specific sequence for FGV [24]. The nucleotide sequences analysis of this 135-bp specific sequence of the 14 isolates showed high similarity to FGV. In addition, the results of nested PCR using the brains were consistent with the histopathological findings, as were those using the feather pulp. Seventeen PCR-positive birds were necropsied and all examined birds were found to have nodular astrocytic proliferation, which was consistent with the lesions in the previous reports [9, 11, 23]. Thus, this short sequence used in this study is appropriate for the detection of FGV.

The variable regions located in the SU sequence in the env gene mainly define the subgroup specificity of ALSVs [3, 6]. In these sequences, mutation and recombination occur [25] and high nucleotide variability among the subgroups is observed [3]. In addition, Chesters et al. [4] showed the possibility that the env gene of ALVs is a determinant of tumorigenesis. Hence, we analyzed the env gene of the isolated viruses for comparison with those of other ALSVs and to clarify the transition and relationship with the neuropathogenesis of FGV. The 14 isolated viruses fell into two groups in the phylogenetic analysis. Substitutions and deletions of nucleotides frequently occurred mainly in the variable region in the env gene of three isolates, Sp_40, Sp_50 and Sp_53. However, no significant differences in histology were observed between the brains infected with Sp_49, Sp_50, Sp_53, U_1 and U_22. Therefore, all iso-
lates are considered FGV mutants and could induce astrogliomas. The common region of env gene between FGV and the isolates may play an important role on neuropathogenicity of FGV.

Phylogenetic analysis based on the env gene indicated that two viruses, avian myeloblastosis-associated virus type 2 (MAV-2) and Rous sarcoma virus Schmidt-Ruppin B, are related to FGV (Fig. 2), although they do not possess the 135-bp sequence specific for FGV in the 3' UTR. MAV-2 was originally isolated as a helper virus of avian myeloblastosis virus and is a replication-competent virus without a viral oncogene. This virus can induce nephroblastoma and osteopetrosis if the virus infect alone [13]. In contrast, Rous sarcoma virus Schmidt-Ruppin B is a replication-competent sarcoma virus with an oncogene, and causes acute connective tissue tumors, e.g., fibrosarcoma. Thus, these viruses have different pathogenicity from that of FGV.

In conclusion, we demonstrated that FGV with a mutation in the env gene apparently spread in chickens of the zoological gardens and these mutants still have astrocytoma oncogenicity in the avian central nervous system. Although the present results were anticipated in some degree and we know the fact that env of ALVs has a high potential for mutation, the actual prevalence rates were greater-than-expected levels. These flocks are useful for clarifying the molecular basis of retroviral neuropathogenicity based on the consecutive analysis from the pathological and virological aspect.

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