

Cutaneous neuro-myofibroblastic sarcoma induced by avian leukosis virus subgroup J in a rooster (*Gallus gallus domesticus*)

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Key words:

avian leukosis, cutaneous sarcoma, *Gallus gallus*, myofibroblastic tumor, rooster

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Received: 23 May 2016

Accepted: 15 August 2016

Abstract:

An adult native cock (*Gallus gallus domesticus*) referred to the aviary clinic with multiple different sizes of round dermal nodules. The bird died few days later, and was then submitted for further evaluation. Macroscopic and microscopic examinations as well as a PCR test were done to identify type and cause of the tumor. In histopathological assessment of biopsy specimen, it consisted of interlacing bundles of fibroblasts that orientated in different directions with plump or elongated spindle shaped nuclei and fairly abundant cytoplasm. At necropsy several large white nodules were implanted in lung and liver. Microscopically the proliferated fibroblastic cells were invaded to both organs, and were similar to those described for skin lesion. The tumor cells had immunoreaction for alpha smooth muscle actin, vimentin and S100 protein, whereas they were negative for desmin and pancytokeratin, suggesting a diagnosis of metastatic neuro-myofibroblastic sarcoma. A PCR test specific for avian leukosis virus subgroup J (ALV-J) confirmed the presence of that virus in tumor specimens. Sequencing and phylogenetic analysis showed a relatively low similarity in the LTR segment (90%) of the studied virus with other ALV-J strains. It might be the first report of cutaneous neuro-myofibroblastic sarcoma, potentiated to metastasis to other organs induced by ALV-J.

Case History

The etiology of neoplastic diseases, specifically those appearing in exotic or wild birds is not well-defined, conversely the oncogenesis in poultry is more commonly identified, in which the most documented causes are infective. Avian leukosis/sarcoma viruses (ALSVs; subgroups A-E and J) related to the oncogenic retroviruses, are involved in the pathogenesis of numerous

classified tumors originated from either mesenchymal or epithelial cells (Hafner et al. 1998; Ono et al. 2004; Ochi et al. 2012; Wang et al. 2013).

Among the ALV-encoded proteins, the envelope glycoprotein (env) on the surface of retroviral particles is the major determinant of the subgroup phenotype, host range and antigenicity. The env gene sequence of the prototype virus of subgroup J, HPRS-103, differs extremely from other ALV

subgroups and has high identity (75%) to env-like sequences of some members of the EAV family of the endogenous avian retroviruses. Moreover, a novel EAV related sequence, designated EAV-HP, demonstrated in the chicken genome, has revealed about 97% homology to that of HPRS-103 env gene (Bai et al., 1995). Multiple copies of EAV-HP elements were present in the genome of all the lines of chickens and the ancestral jungle fowl (Smith et al., 1999). The above mentioned researches suggest that the emergence of ALV subgroup J might be the result of recombination with env-like sequences of avian leukosis E family. In the present study, the first report of neuromyofibroblastic sarcoma in chicken induced by ALV subgroup J in Iran has been described.

Clinical Presentation

A 2-year-old cock (*Gallus domesticus*) referred to birds' clinic in the School of Veterinary Medicine of Lorestan university was examined with raised dermal multicentric nodules, locating on both left and right knee region, left lateral shank region, and right radial region of wing from single to multiple, clustered together, but the largest singular mass was 3.5 cm in diameter. The bird was being kept in a free-range grazing environment with several poultry, however, others had no similar signs. The firm nodules had no attaching to their underlying tissue, and it was possible they originated from the skin.

The biopsy sample obtained from knee was presented for pathologic examination. It was fixed in 10% neutral buffered formalin, routinely processed and stained with hematoxylin and eosin (HE).

Three weeks later the general condition of bird deteriorated progressively and it

died before returning to clinic.

Diagnostic Testing

Macroscopic examination: In addition to nodules which were described previously, a few small skin nodules were also unveiled in post mortem evaluation, deeply embedded and firmly adherent to their adjacent subcutaneous fascia and muscles. The nodules were well-circumscribed, firm with gray/white color and smooth on cut surface. Of the internal organs, only liver and lung were affected with white spherical nodular growths, fairly invariable in size (Fig 1 and 2).

Microscopic examination (Biopsy specimen histopathology): The lesion was well-circumscribed but it had no true capsule. The cells were either arranged in broad interwoven bundles of highly cellular fibroblast-type cells, with moderately collagenous fibrous tissue, or to a lesser extent were present in loose-ground substance like- whorl pattern. Most of them had oval to elongated vesicular nuclei which contained one or two prominent nucleoli. Although many were markedly pleomorphic, nuclear hyperchromatism was occasionally present. The cells had considerable amounts of cytoplasm, but their boundaries were ill-defined. Moreover, moderate population of cells with typical eosinophilic granules were dispersed among the neoplastic cells.

Mild acanthosis, but extensive hyperkeratosis were striking features of epidermal layer. It was characterized by the formation of intraepidermal spongiosis of keratinocytes which differed from micro-size to moderate. Basophilic stained colonies of bacteria were striking in epidermal layer; however, inflammatory cell did not increase.



Figure 1. Multiple white nodules are implanted in the lung parenchyma.

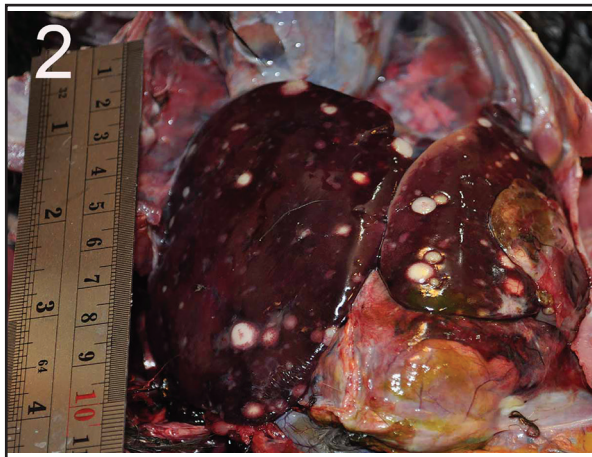


Figure 2. Multiple white nodules are implanted in the liver.

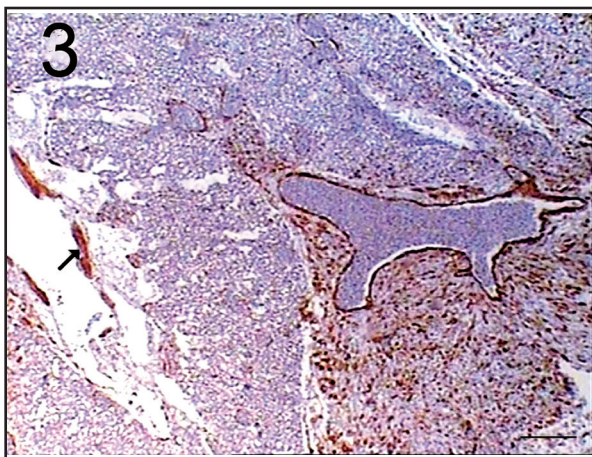


Figure 3. Pulmonary positive immunoreaction of neoplastic cells to alpha smooth muscle actin. The smooth muscle cells in the vascular wall as well as in the bronchus (arrow) are also immunolabeled consistently as internal positive control. (200µm).

Post mortem histopathology: Microscopic examination in liver and lung was

exactly the same as the description stated for subcutaneous mass, in which tumor cells invaded their parenchyma.

Immunohistochemistry: The following antibodies were applied in appropriate dilutions on tissue targets: vimentin (Dako, Glostrup, Denmark), S-100 protein (Dako), neuron-specific enolase {NSE (Dako)}, pan-cytokeratin (Dako), alpha-smooth muscle actin (Dako) and desmin (Dako) by use of Envision kit (Dako). Moreover, S-100 and alpha-SMA were also used on normal skin of chicken. Neoplastic cells were strongly immunoreacted for alpha-smooth muscle actin, vimentin, S100 and NSE (Fig.3-6), but they had no reaction to desmin and pan-cytokeratin. The smooth muscle cells of blood vessel walls were considered as internal positive controls for actin and vimentin, but control skin of chicken was not immunolabeled at all to alpha-SMA and S100. S100 and NSE antibodies had cross reaction with intestine and brain of chicken respectively and for pituitary adenocarcinoma of budgie. Based on histopathology results, the type of tumor was neuro-myofibroblastic sarcoma.

Polymerase chain reaction. RNA was extracted from tumor tissues (liver, lung and skin) by using RNeasy Mini Kit, according to the manufacturer's instructions (Qiagen, Germany), and was stored at -80 C.

Specific reverse transcription Polymerase chain reaction (RT-PCR) for subgroup J of ALV was performed directly on tumor tissues. RT-PCR analysis with the primers, forward primer DU5F 5'-GGGCGGG-GCTTCGGTTGTA-3' and reverse primer DU5R 5'-TCGCTCATGCAGGTGCTC-GTAGTT-3' was performed (Zavala et al. 2002). These primer sets for ALV-J amplified the 517 base pair (bp) segment specific

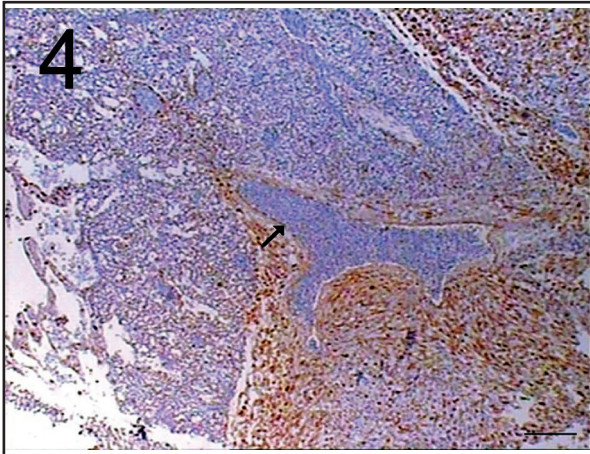


Figure 4. The tumor cells in lung are displaying positive staining for vimentin. The rim of large vein is unevenly stained (arrow). (200µm).

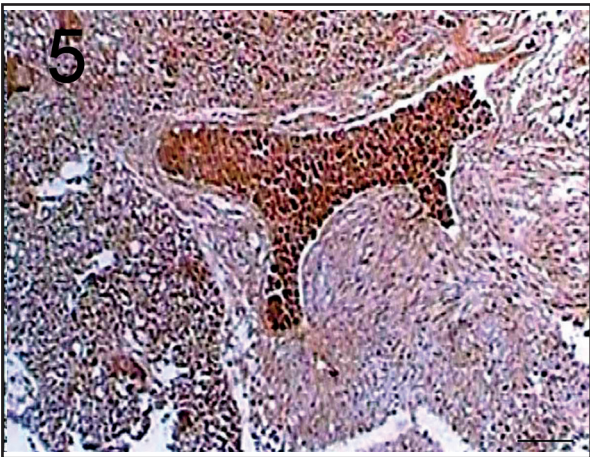


Figure 5. expression of S100 protein in Schwann tumor cells, those positive cells are more dispersed than alpha-sma and vimentin. The red blood cells are also false positive stained.

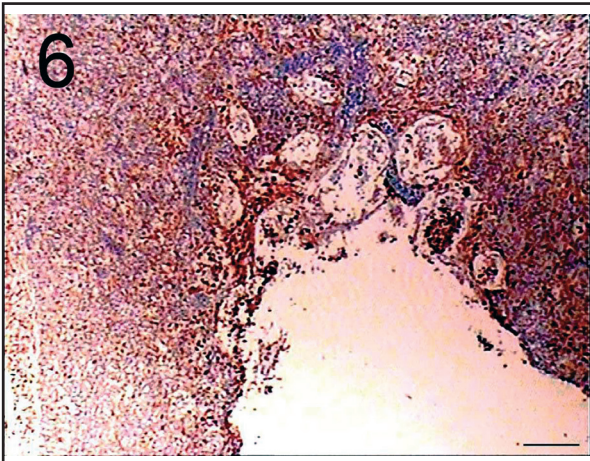


Figure 6. immunopositive neoplastic cells to NSE, are more condensed around parabronchus. (E-J; 200µm).

for the LTR region of ALV subgroup J. For

positive control, positive DNA was kindly provided by Dr. K. Venugopal and for negative control DEPC treated water was used. The PCR mixture was heated at 93C for 3 minutes, then subjected to 35 cycles of PCR amplification (93C for 10 seconds, 54C for 10 seconds, and 72C for 20 seconds) and finally at 72C for 5 minutes using a T100 TM Thermal Cycler (Bio-Rad, USA).

In another PCR for ALV, the primers' H5 (5-GGATGAGGTGACTAAGAAAG-3) and AJ1 (5-ATGAACGGCCCATTC(T/C) CCTATTCC- 3) amplified the hyper variable region in the env gene of ALV subgroups A to E (Venugopal et al. 2008). The PCR mixture was heated to 94 C for 1 minute and then subjected to 30 cycles of PCR amplification (94C for 60 seconds, 60C for 60 seconds, decreasing to 48c during 30 cycles, and 72C for 2 minutes, followed by an extra 10 minutes at the end of the last cycle), which produced 1758 bp DNA fragments from the tumors of the affected chicken. To determine any role of Marek's disease virus 1 (MDV1) in tumor lesions, PCR was performed on the same materials (Handberg et al. 2001). The positive control was positive DNA, obtained from Dr. K. Venugopal.

PCR products were detected after 1.5% agarose gel electrophoresis and were purified by the Gene JET Gel Extraction Kit (Fermentas, Canada), according to the manufacturer's instructions. The purified DNA fragments were cloned into the pTZ57R/T cloning vector (Fermentas, Canada). Three clones of each fragment were sequenced using M13 forward and M13 reverse primers (Promega, USA) at Sequetech Co. Ltd., USA. Nucleotide sequence alignment and phylogenetic analysis were carried out by the Clustal W method in MegAlign of DNASTAR program (Madison, WI). The

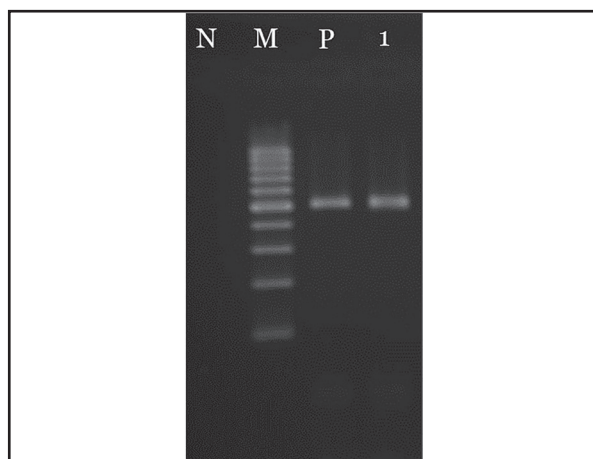


Figure 7. PCR result for detection of ALV subgroup J. Lane N: Negative control; M: 100 bp Marker (Fermentas, Canada); Lane P: Positive control; Lane 1: tumor sample.

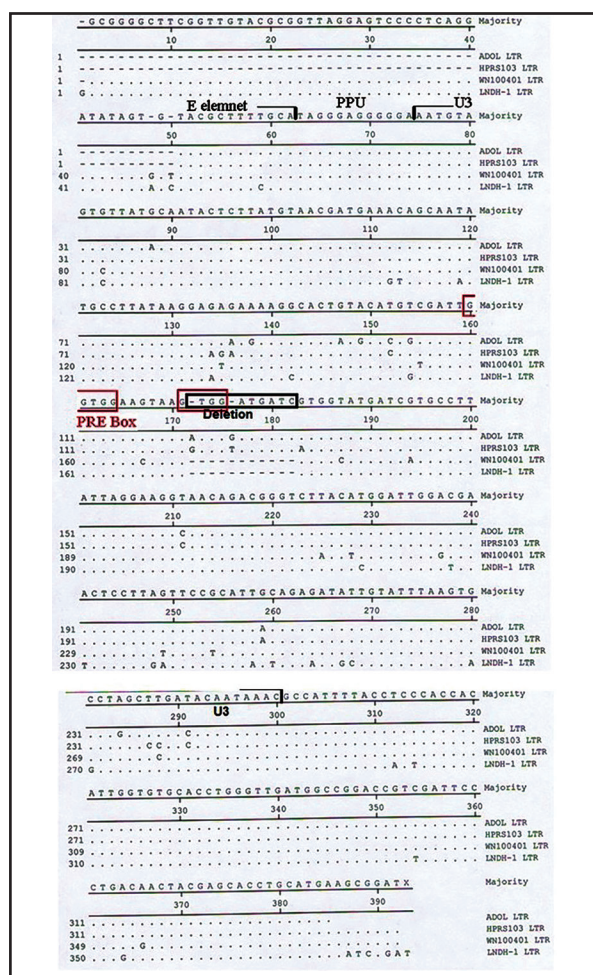


Figure 8. Sequence Comparison of the LTR of ALV subgroup J strains ADOL-7501, HPRS-103, WN100401 and the sequence obtained in this study (LNDH-1). Dashes are equal to homology among compared sequences; substitutions are shown by the nucleotide symbol in related positions. Deletions and PRE boxes are showed with black and red triangles, respectively.

sequences determined in this study are available in GenBank (accession numbers KT326192 and KT326193).

Using primers specific for ALV subgroup J (DU5F and DU5R), a positive result could be detected in all examined tumor tissues (Fig. 7), although the MDV-1 specific sequence was detected in positive control but not in the tissues. A segment of about 1.8 bp of env gene was detected in the other PCR test.

Molecular characterization of LTR. The nucleotide sequence of the LTR region of the studied ALV subgroup J was compared with strains ADOL-7501, HPRS-103 and WN100401 (Fig. 8). A total number of 26 base substitutions were found in the LTR region of the studied ALV subgroup J in comparison to the prototype strain HPRS-103.

The sequence analysis of LTR of the studied virus revealed that its U3 region has only 215 bp, because of an 11 bp deletion. The R and U5 regions of the studied ALV subgroup J were conserved compared with other ALV subgroup J isolates.

Some sequences regulating transcription of the ALV have been identified in the U3 region of the LTR, including CAR_G, Y, and PRE, C/EBP, CAAT, and TATA boxes (Gao et al. 2012). In most ALV subgroup J isolates (including HPRS-103 and ADOL-7501), there are two PRE boxes (GGTGG motif), flanking the nucleotide sequence AAGTAA. One of the PRE boxes was omitted at position 171 to 174, for the deletion (Fig. 8).

Phylogenetic analysis and sequence similarity. Based on results of phylogenetic analysis, the LTR sequence of the studied virus belonged to a new branch (Fig. 9) and has the least (82.2%) and the most (88.1 %) sequence similarity with GD1109

		Percent Identity													Divergence	
	1	2	3	4	5	6	7	8	9	10	11	12	13			
1		91.0	95.8	94.5	91.0	92.6	86.7	89.1	95.3	93.2	92.3	90.0	89.6	1	ADOL LTR	
2	9.0		92.1	93.2	95.0	96.6	85.1	92.1	92.2	91.4	98.5	89.2	90.9	2	GD1109 LTR	
3	4.3	7.7		97.8	92.0	93.7	89.6	91.1	99.0	95.6	93.1	91.8	94.1	3	HPRS103 LTR	
4	5.7	7.1	2.2		92.4	93.6	86.7	94.6	98.3	95.1	93.6	93.2	93.2	4	JS nt LTR	
5	9.7	4.5	8.6	8.1		98.7	83.7	87.7	91.9	90.7	97.0	89.0	88.0	5	JS09GY2 LTR	
6	7.9	3.5	6.6	6.7	1.3		88.3	92.4	93.2	91.7	98.2	90.1	93.2	6	JS09GY3 LTR	
7	12.5	16.3	13.0	14.5	15.6	15.3		86.4	85.8	86.0	86.4	89.6	88.9	7	LNDH-1 LTR	
8	8.6	7.7	6.5	5.6	9.6	8.0	14.4		90.2	92.4	92.1	90.2	95.1	8	NHH LTR	
9	4.9	7.6	1.0	1.7	8.7	7.2	12.4	6.7		95.6	92.9	91.9	89.2	9	NX0101 LTR	
10	7.2	9.3	4.6	5.1	10.9	9.6	15.1	8.1	4.6		91.7	91.4	91.2	10	SD07LK1 LTR	
11	8.2	1.6	7.3	6.7	3.1	1.9	15.8	8.4	7.5	9.6		90.1	92.5	11	sdau1002 LTR	
12	10.9	11.6	8.8	7.5	12.1	11.1	10.6	9.6	8.7	9.6	11.1		89.3	12	SVR807 LTR	
13	8.9	9.1	6.1	7.2	9.2	7.2	13.6	5.1	7.8	9.4	8.3	11.3		13	WN100401 LTR	
	1	2	3	4	5	6	7	8	9	10	11	12	13			

Figure 9. Comparison of the nucleotide sequence identity of the LTR gene of LNDH-1 and other ALV-J strains.

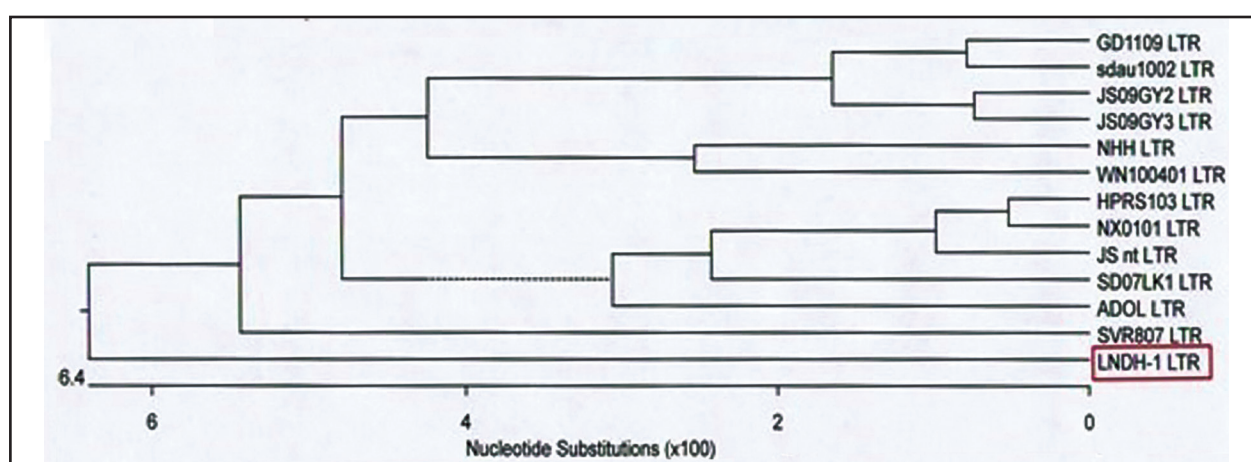


Figure 10. Phylogenetic analysis of the LTR region of ALV subgroup J isolates (including the studied ALV-J).

and WN100401 strains, respectively (both isolates from China). In the phylogenetic tree (Fig. 10) of the LTR, the studied virus (LNDH-1) is placed in a separate branch.

A BLAST search revealed that the LTR and 1.8 bp env fragments of the studied ALV subgroup J were 90% and 99% homologous to the region of LTR of the ALV subgroup J isolate WN100401 and env of the endogenous virus strain ALVE-B11, respectively (GenBank accession numbers KC11043 and KC610517).

Assessments

Alpha-smooth muscle actin is one of actin isoform, found basically in smooth muscle cells, but it exists in largest amounts

within vascular smooth muscle cells and has a function in development of fibrogenesis (Cherng and Young, 2008; Kawasaki et al. 2008). The expression of alpha-SMA occurs specifically in activated myofibroblasts, which differentiate from cutaneous fibroblasts and also contains some biological characteristics of smooth muscle cells (Cherng and Young, 2008; Elberg et al. 2008; Nakatani et al. 2008). These cells are present in tissue injury for wound repair by its contraction and their appearance and proliferation are provisional and dependent on exposure to chemical substances or cytokines (Park et al. 2007; Cherng and Young, 2008). However, after repair completion, these cells disappear through apoptosis (Darby and Hewitson, 2007; Cherng

and Young, 2008). Alpha-SMA is present in mouse subcutaneous fibroblasts (Storch et al. 2007; Cherng and Young, 2008), but in present study it was absent in normal skin tissue of chicken. Therefore, it might be concluded that presence of neoplastic myofibroblasts is a process in response to permanent mutated fibroblasts which are motivated by ALV-subgroup j.

Myofibroblastic tumors which are also designated as myofibrosarcoma and myofibroblastic sarcoma are documented in a few reports in veterinary literature (Bell et al. 2011, Newman et al., 1999, Silva et al., 2012).

According to the case, in one study the authors concluded that positive labeling of alpha-smooth muscle actin and vimentin and negative immunoreactions for desmin and S100 protein were suggestive of myofibroblastic sarcoma in the limb of a horse (Silva et al . 2012). In another report in a horse, desmin was also positive, similar to alpha-SMA and vimentin but negative for S-100 which the authors believed that the originated tumor based on diagnostic criteria of human being was myofibroblastic sarcoma and such tumors are consistently negative to S-100 (Newman et al. 1999).

According to the investigation of 10 feline orbital tumors, Bell et al. (2011) realized that all myofibroblastic sarcomas immunostained with smooth muscle actin and vimentin, whereas interestingly, in contrast to previous reports all tumors reacted to S-100 which is probably related to origination of cells from neural crest in facial mesenchyme. As mentioned, other tumors of myofibroblastic sarcomas whether in animal or human being were negative for S-100, therefore, presumably its expression in rooster skin tumor pertains to two distinct

lines of tumor cells.

Among tumor types the prevalence of fibrosarcoma in chickens is significantly less than other tumors which were exposed by infected ALV-J(Cheng et al. 2010). The low incidence of connective tissue neoplasms in chickens indicates that the origin and histogenesis of such lesions are not defined exactly and their appearance sporadic (Strafuss and Ladds, 1970; Reece, 1996).

Based on the PCR results, genome of the studied rooster has been affected by an ALV of subgroup J. In the tumor tissues, MDV proviral DNA was not detected using PCR, but a 1.8- kbp fragment of the env region was amplified and it was most similar to ALVE-B11. ev loci are inheritable proviral elements and ubiquitously identified in chicken cells. Normally, few or no endogenous ALVs (EAV) are expressed from ev loci in chickens. The reason behind this inefficient viral particle production is that the locus has a (+1) frame shift mutation in the pol RT- β region, which results in a truncated RT- β subunit and integrase (Johnson and Heneine, 2001). Hatai et al. (2008) isolated a strain of ALV (TymS_90) from layer chickens with fowl glioma that was a recombinant ALV derived from ev-1 and other strains of avian leukemia and sarcoma viruses. Although such hypothesis could be assumed for the ALV subgroup J sequenced in the present study, it could not be clarified whether the detected fragment was derived from an EAV or such a recombinant ALV. More detailed study, using primers specific to ALV subgroup J should be performed in the future.

The pathogenicity and oncogenesis potential of ALVs are not merely concerned with changes in the env gene, although they are the most important known factors. Two

non-coding segments in the genome of ALV subgroup J, including U3 region of LTR and E element, also have a significant role in the pathogenicity of the virus (Hue et al. 2006; Gao et al. 2012). The LTR sequences of the ALV subgroup J strains have been previously compared (Gao et al. 2012). The phylogenetic analysis indicated that the LTR sequence of the studied isolate has approximately low homology with each of the other strains presented in the phylogenetic analysis. Twenty-six nucleotide substitutions in the U3 region were found in the studied isolate. Other scientists also mentioned that the U3 region of LTR evolves rapidly (Gao et al. 2012). A deletion of 11 bp was seen in our studied ALV subgroup J. Such a deletion is relatively rare among ALVs of subgroup J and is seen in only some Chinese strains, e.g. WN100401 (accession number HQ271447). The U3 region of the LTR contains transcriptional regulating elements and determines both the level of viral transcription and the oncogenic potential of ALVs (Gao et al. 2012). The nucleotide deletions in the U3 region of LTR in our studied virus caused omission of PRE box (Fig. 8).

This report is the first of ALV subgroup J, obtained for a chicken's tumor in Iran. The LTR region of the ALV subgroup J isolate, studied in this article, formed a separate branch from the prototype strain HPRS-103, a strain from the US (ADOL-7501) and other Chinese ALV subgroup J strains, including those isolated from broiler or layer chickens (Fig. 10). According to the sequence identity, the homology among LTR region of the studied ALV subgroup J and other ALV-J strains is relatively low (maximum 88.1%) (Fig. 9). Although it is not determined whether this information is related to changes in functional characteristics

of LTR gene, some interesting points on the molecular characteristics of the studied ALV subgroup J isolate were revealed. Such information together with more comprehensive future studies on the virus will contribute to a better understanding of the genetic differences of the studied ALV subgroup J with other countries' isolates.

Acknowledgements

We gratefully acknowledge the Deputy of Research Office of Lorestan University for their financial support.

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نورومیوفیبروبلاستیک سار کومای جلدی متاستاتیک ناشی از لکوز J در یک قطعه خروس بومی: گزارش موردی

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(دریافت مقاله: ۳ خرداد ماه ۱۳۹۵، پذیرش نهایی: ۲۵ مرداد ماه ۱۳۹۵)

چکیده

تومورهای ناشی از لکوز J در پرندگان فراوان است ولی گزارشات اندکی از آن در ایران ارائه شده است. در این مطالعه یک مورد تومور پوستی ناشی از لکوز J در یک خروس بومی بررسی شده است. یک خروس نژاد لاری ارجاع شده به کلینیک دامپزشکی دچار تومورهای گرد پوستی در قسمت‌های مختلف بدن شده بود. پرنده پس از چند روز تلف شد و برای بررسی بیشتر مورد کالبد گشایی قرار گرفت. در بررسی هیستوپاتولوژی نمونه بیوپسی دسته‌های فیبروبلاست با جهت گیری‌های مختلف مشاهده شد که هسته آنها حالت کشیده پیدا کرده و حاوی مقدار نسبتاً زیادی سیتوپلاسم بود. در کالبد گشایی ندول‌های بزرگ سفید رنگ در ریه و کبد مشاهده گردید. در بررسی میکروسکوپی سلول‌های فیبروبلاست تکثیر شده مشاهده شده در این دو اندام مشابه ضایعات پوست بودند. در ایمونوهیستوشیمی بافت‌های تومور با اکتین عضله صاف آلفا و پروتیین S۱۰۰ واکنش نشان دادند ولی با دسمین و پانسیتوکراتین واکنش نشان ندادند که حاکی از سار کومای نورومیوفیبروزی متاستاتیک بود. نتایج آزمون PCR برای بیماری مارک منفی و برای لکوز J مثبت شد. آنالیز فیلوژنیک حاکی از شباهت نسبتاً کم قطعه LTR (۹۰٪) و ویروس مطالعه شده با دیگر سویه‌های لکوز J بود. این مورد اولین گزارش تومور از نوع مذکور ناشی از لکوز J در ایران است.

واژه‌های کلیدی: لکوز J، سار کومای جلدی، گالوس گالوس، تومور نورومیوفیبروبلاستیک، خروس بومی

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