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Mutation and up-regulation of TP53 in ovine pulmonary adenocarcinoma lung cells as a model of human lung cancer

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Abstract

Ovine pulmonary adenocarcinoma (OPA) is a model of human lung cancer and fatal viral disease that causes neoplasia in sheep respiratory cells. In the current study, 986 lung samples was inspected in the slaughterhouse, and finally twenty OPA lung organs were clinically diagnosed and five healthy lung organs were assigned as the control sample. Three SSCP patterns were detected for the affected lungs animals in comparison with the healthy lungs. In addition, sequencing results indicated three different single point mutations in exon 4 of TP53 within infected lungs, whereas no mutations were observed in exon 9 of this gene. Real-time PCR results showed up-regulation of the TP53 gene in all the infected lung cells compared to healthy cells. There was significant correlation between the mutations in exon 4 and OPAand can be used as a useful tool in determining the mechanism of lung cancer.

Introduction

Ovine pulmonary adenocarcinoma (OPA) also known as Jaagsiekte disease is one of the chronic respiratory diseases which involves the lung of sheep, and rarely of goats. The OPA is used as animal model for human pulmonary adenocarcinoma and bronchial-alveolar cancer (BAC).[1] OPA is caused by beta-retrovirus or jaagsiekte sheep retrovirus (JSRV) that has special tropism to differentiated epithelial cells and secretory epithelial cells of the lungs.[2] It is identified by pathological lesions and PCR method.[3] It is the only known virus that causes progressive pulmonary adenocarcinoma which involves the whole lung.[2-4] Mutagens associated with this virus have been implicated in causing genetic changes such as mutations in p53 gene.[5]

One of the mechanisms of JSRV-induced cancer is implemented when the virus needs to attach a virus-coated protein to a cell surface receptor molecule called hyaluronidase 2 (HYAL 2) in order for the virus to enter the cell.[6] HYAL 2 is, in fact, a protein linked with the glycosylphosphatidylinositol and is expressed in many tissues and cells. The local formation of viral envelope in target cell membrane is a good means for mixing with several cellular protein kinases.[6] This continuous activity is the signaling pathway for protein kinase which is the stimulator of cellular proliferation and transformation. All these activities combined together have an important role in carcinogenesis.[7] In literature, the role of p53 has been reported as unclear and the investigation of this kind of mutation in lung cancer was suggested earlier.[8] The global spread of OPA creates diverse and often significant economic losses and this disease was introduced by the World Health Organization for Animal Health[9] as an important disease of sheep throughout the world.[10] The exact diagnosis of the disease in infected animals often requires histopathologic examination and autopsy.[11] Immunohistochemistry studies indicated the similarity of human pulmonary adenocarcinoma (HPA) and OPA. In addition, because of the pathological and epidemiological similarities between OPA and human pulmonary adenocarcinoma and bronchial-alveolar cancer (BAC), OPA has been identified as a unique animal model for humans with HPA and a valuable model for anti-cancer examinations.[4,12]

Exploring the genetic changes in the advanced levels of the disease helps understand new factors useful for treatment of HPA. On the other hand, microscopic and

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pathologic examinations based on morphologic features along with an investigation of tumor promotion and initiation is useful in HPA type exploration, prognosis can be diagnosed.[13]

Environmental factors such as smoking and viruses play a direct role in human lung cancer. Bronchial-alveolar cancer (BAC) is a type of lung cancer that does not affect small cells, known as non-small cell lung cancer (NSCLC). There is no information about the outbreak rate and frequency of this disease.

The BAC is a pattern of adenocarcinoma which can be divided into two forms of mucinous and non-mucinous, the latter lacking pleural-vascular-stromal invasion.[14] In this pattern, neoplastic cells are seen as developing form in a single-layer manner in the respiratory wall and pathway, and alveolar region.[15] According to this pattern, in the non-mucinous form, either Clara cells or pneumocyte types 2 develop in alveolus wall, similar to the OPA.[16] Studies have shown that more than 25.00% of lung cancers are of the BAC type.[17]

Although there is no single, unique animal model for human diseases, there are enough similarities recorded between OPA and some HPA forms.[18] In order to support this hypothesis, it can be claimed that the understanding OPA at molecular level provides a logical framework for understanding some aspects of pulmonary carcinogens.[19]

In sheep, the TP53 gene composed of 11 exons, containing 12905 bp nucleotides, and located on the chromosome 11.[20] The TP53 gene product is named tumor protein p53.

In the normal cell, mouse double minute 2 homolog (MDM2) molecule attaches to p53 and inactivate it.[21] In the DNA damage or cellular stress, MDM2 separated from p53 protein and p53 production increased in the cell. Then the activated p53 binds to the DNA and increases the expression of several genes including the WAF1 / CIP1 that encodesP21 molecules and stimulates another gene that is responsible for the production of protein P21.[22] Finally, P21 protein binds to cdk2 (cell division stimulating cyclin-dependent kinase 2 interacting protein) and makes a complex with cdk2. This complex inhibits of the cells to entry to the G1/S phases of the cell division.[23] Mutated TP53 gene cannot attach to DNA, thus P21 protein cannot be made for stopping the cell division, therefore, the cells undergone division and the tumor is formed.[24]

Research on OPA is crucial as a model of HPA research due to its neoplastic nature, long course of disease and economic losses. The results of an immunohistochemical study suggest that a JSRV-like virus may infect humans.[18]

With respect to the high rates of mutation in TP53 gene in different cancers, especially lung cancer, there is more probability that this mutation exists in OPA. Previous studies have been performed on the polymorphism of most TP53 exons with the exception of exons 4 and 9. The aim of the present study was to investigate relationship between mutation of exons 4 and 9 and gene expression of TP53 in infected pulmonary tissues with OPA.

Materials and Methods

Sampling and histopathology examination. A total of 986 ovine lungs were grossly examined for OPA lesions in Tabriz industrial slaughterhouse and 20 samples of affected lungs were transferred to the pathology laboratory. In order to prepare samples for pathology and molecular study, five samples from healthy lungs were used as the control group. Samples were taken from the affected parts and border areas and fixed in 10.00% neutral buffered formalin. After 48 hr, formalin in the sample containers was renewed. In addition, 50.00 - 100 mg of affected lung tissue was put in RNA-later (Sigma, St. Louis, USA) and PBS (Sigma) buffers for RNA and DNA extraction, respectively, and stored at – 20.00 ˚C. Preparation of histopathological sections were performed according previous studies.[23]

Extraction of DNA and polymerase chain reaction (PCR). DNA was extracted from clinical samples as described by Campos and Gilbert[25] with some modifications, and was kept at – 20.00 ˚C. Quality and quantity of extracted DNA were measured by NanoDrop (Thermo Fisher Scientific, Germany) and 1.00% gel agarose electrophoresis. For amplification of ovine TP53 gene two primers were used as described by Dequiedt *et al.*[26] The PCR reaction was performed in a total volume of 25.00 µL and consisted of master mix (12.50 µL), DNA sample (100 ng), forward primer (10.00 pmol), reverse primer (10.00 pmol), and DNase free water to reach final volume. Both exons were amplified using the following PCR conditions of 95.00 ˚C for 5 min (1 cycle), followed by 40 cycles of 94.00 ˚C for 1 min; 65.00 ˚C for 30 sec;72.00 ˚C for 45 sec, and 1 cycle of final extension at 72.00 ˚C for 10 min using a thermo cycler (Bio-Rad, Hercules, USA).

Single-strand conformation polymorphism (SSCP). The SSCP analysis detects different sequences through electrophoretic mobility differences. DNA that contains a sequence mutation (even changed a single base pair) has a measurable mobility difference compared to wild type DNA when subjected to non-denaturing conditions. The PCR products were visualized using electrophoresis using 1.50% agarose gel and 3.00-5.00 µL of these products were used for SSCP technique on 7.00% acrylamide gel. Materials and quantities required for the preparation of 7.00% SSCP gel were as follows: 8.00 mL of a 30.00% solution of polyacrylamide gel (Merck Millipore, Darmstadt, Germany), 23.00 mL of distilled water, 3.00 mL of TBE 10X, 200 µL of a 10.00% ammonium persulfate solution (Merck Millipore), 50.00 µL of the catalyst N, N,N,N-

tetramethylethylenediamine (TEMED; Merck Millipore). Five microliter of PCR products was mixed with prepared loading buffer (95.00% formamide with 6X loading dye) to unwrap the double-stranded PCR product. Then, the mixturewas centrifuged and denatured at 95.00 ˚C for 10 min and immediately placed on ice. The electrophoresis was performed in a vertical electro-phoresis tank or unit (Bio-Rad) with 120 V for 390 min. Silver staining was performed for spotting. The required materials and staining protocols were conducted as follows: 10.00% ethanol and 1.00% acetic acid (Merck Millipore) as stabilizer solution, 0.10% silver nitrate (Merck Millipore) as stain solution, 1.50% sodium hydroxide (Merck Millipore) and 0.10% formalin as appearance solution, 0.75% sodium bicarbonate (Merck Millipore) as stop solution, finally the gels were kept at 1.00% acetic acid as preservative solution.[27]

Sequencing. The PCR products (15.00 µL) of each different pattern with forward (10.00 µL) and reverse (10.00 µL) primers were sent to Marcrogen Co. (Seoul, South Korea) for two times sequencing on one sample.

RNA extraction and cDNA synthesis. Tissue Total RNA Mini Kit (FavorPrep™, Kaohsiung, Taiwan) was used for extraction of total RNA according to manufacturer protocol and extracted RNA was stored at – 80.00 ˚C. The 0.10% diethylpyrocarbonate (DEPC) Solution (Fermentas, Burlington, Canada) was used to inactivation of the RNAase enzyme in the all equipment. Extracted RNA was treated with DNase I enzyme (Fermentas) to avoid contamination with the genomic DNA. In order to assess the quality of RNA, 2.00% agarose (Bioneer, Daejeon, South Korea) gel electrophoresis method was used. Moreover, quantitative assessment of RNA was measured using a spectrophotometer, in the range of 260 nm absorption. Then, cDNA was synthesized using cDNA Synthesis Kit YTA (Yekta Tajhiz Azma, Tehran, Iran) according to the manufacturer's protocol.

Real-time PCR. For amplification of mRNA of ovine p53 one pair primers were designed based on the sequence with gene bank accession number (FJ855223.1) via Primer3 software and checked with OligoAnalyzer 3.1. To enhance the qPCR production accuracy, the primer was designed in such a way that its forward was placed in exon 9 and parts of its reverser in exon 10. As well as; Forward-5'- CACTGCCCAGCAG CACCAGC -3', Revers-5'- CGCCCAC GGATCTGAAGAGTG -3'.GAPDH primer (forward: 5'-GTCCGTTGTGGATCTG ACCT-3 and reverse: 5'-TGCTGTAGCCGAATTCATTG-3') was used as housekeeping gene as previously described.[28] The SYBR green method was applied as 5.00 µL of Mastermix (FavorPrep™), 1.00 µL of synthesis cDNA, 20.00 pmol of forward primer and 20.00 pmol of reverse primer with nuclease free water was subjected to PCR-amplification with 20.00 µL final reaction by thermo cycler (Rotor-Gene Q; Qiagen, Hilden, Germany). Advised program for real-time PCR was followed as: 95.00 ˚C for 5 min (1 cycle), followed by 40 PCR cycles 94.00 ˚C for 30 sec, 65.00 ˚C for 30 sec, 72.00 ˚C for 35 sec, and 1 cycle for final extension step at 72.00 ˚C for 10 min, at last, storage step at 4.00 ˚C for 10 min. All expression levels were normalized to GAPDH (i.e., housekeeping gene: HKG) in each well. The comparative C_T (threshold cycle) method,[29] that uses an arithmetic formula of $2^{-\Delta\Delta CT}$, was applied to calculate the amount of target gene normalized to an endogenous reference (HKG) using data generated during the PCR experiment as below;

$$\Delta C_T = C_{T\,Target\,gene} - C_{THKG}$$

$$\Delta\Delta C_T = \Delta C_{T\,Sample} - \Delta C_{T\,Calibrator}\ Fold\ change = 2^{-\Delta\Delta CT}$$

Statistical analysis. Statistical analysis of gene expression data was performed using Relative Expression Software Tool (REST)[29] and Excel (version 15.0; Microsoft Corporation, Redmond, USA) was used for draw charts.

Results

Pathological findings. The affected lungs were heavy and contained multifocal nodules or local extensive mass that were firm and gray (Fig. 1) with foamy catarrhal secretion on cut surface especially within airways. Metastases to regional lymph nodes such as bronchial and mediastinal lymph nodes were observed. Histopathologic examination of lung in the affected area revealed well-differentiated pulmonary carcinoma with lepidic and papillary patterns (Fig. 2A).

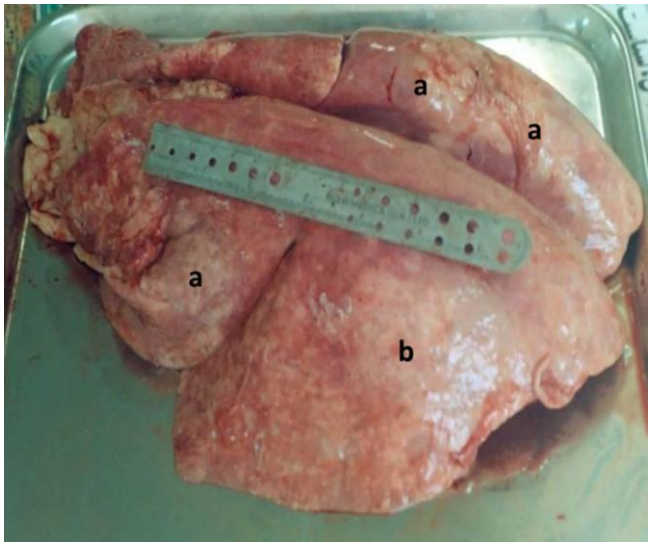

Fig. 1. Macroscopic view of the lung suffering from ovine pulmonary adenocarcinoma (OPA). An affected lung with multifocal nodules (a) and a light gray, local extensive mass (b) (the diaphragmatic lobe of left lung).

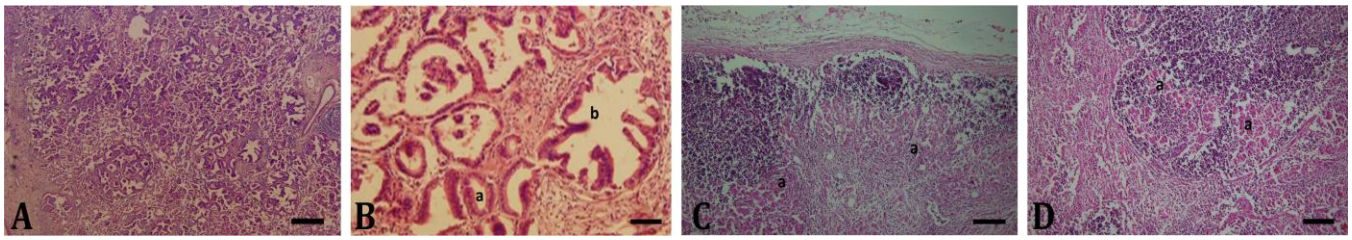

Fig. 2. Microscopic view of the lung suffering from OPA using Hematoxylin and Eosin stain. **A)** Histopathologic section of OPA, well-differentiated pulmonary carcinoma with lepidic and papillary patterns (bar = 300 µm). **B)** Alveoli (a) and terminal brochiol (b) are lined with cuboidal or columnar neoplastic epithelial cells and in papillary pattern, papillary projections into the alveoli and bronchioles are observed in lepidic pattern (bar = 60 µm). **C** and **D)** Neoplastic cells were observed in parafollicular zone of bronchial and mediastinal lymph nodes (bars = 300 µm).

In lepidic pattern, alveoli were lined by cuboidal or columnar neoplastic epithelial cells and in papillary pattern, papillary projections into the alveoli and bronchioles were seen (Fig. 2B). Most of the alveoli in affected areas had significant large foamy alveolar macrophages. Also, neo-plastic cells were observed in parafollicular zone of bronchial and mediastinal lymph nodes (Figs. 2C and 2D). Pathologically confirmed cases were referred to molecular studies.

PCR products. PCR products including 310 bp and 193 bp fragments respectively for exons 4 and 9 successfully amplified by used primers (Fig. 3).

SSCP results. The result of SSCP did not show the different SSCP pattern among PCR products of exon 9 in both affected and healthy samples showed the same pattern (Figs. 4A and 4B). In PCR products of exon 4, we found three types of genotype patterns of SSCP in affected samples in comparison to healthy samples (Fig, 4C). These different patterns named; pattern A (which observed in five affected samples: 25.00% frequency), pattern B (which observed in six affected samples: 30.00% frequency), and pattern C (which observed in nine affected samples; 45.00% frequency).

Sequencing results. Sequencing results (Sequencing Service–Marcrogen, Korea) of three different observed SSCP patterns (A, B, and C) confirmed that there were some sense mutations in the exon 4 of infected samples. Exon 4 of ovisaries tumor suppressor TP53 gene (AF525302.1) contains 105 bp nucleotides with 35 amino acidsIn the pattern A, there was a single nucleotide mutation in 14th amino acids (CCT: Proline converted to CGT: Arginine). In the pattern B, there was a nucleotide mutation in 27th amino acids (GAA: Glutamine converted to GGA: Glycine). In the pattern C, it was observed a nucleotide mutation in 16th amino acids (AAC: Asparagine converted to TAC: Serine). The data was shown in supplementary data.

Real-time PCR. cDNA was synthesized on extracted RNA and real-time PCR carry out on synthesized cDNA. Double measuring gene expression in each sample was conducted. Each sample was divided on average until the relative gene expression for each sample obtained by mean ± SEM. Results and amplification curves of exon 9 TP53 and GAPDH genes by using the real-time showed that amplification was successful. For statistical analysis, it was done the normalization method using obtained datasamples and internal control (housekeeping gene) data.[30] Over expression of TP53 gene was obtained in the infected samples to OPA compared to the healthy samples. Relative expression of twenty infected samples which divided in three types SSCP pattern (five samples showed A pattern, six samples showed B pattern, and nine samples showed C pattern) compared to five healthy samples which are showed monomorphic pattern (Fig. 4).

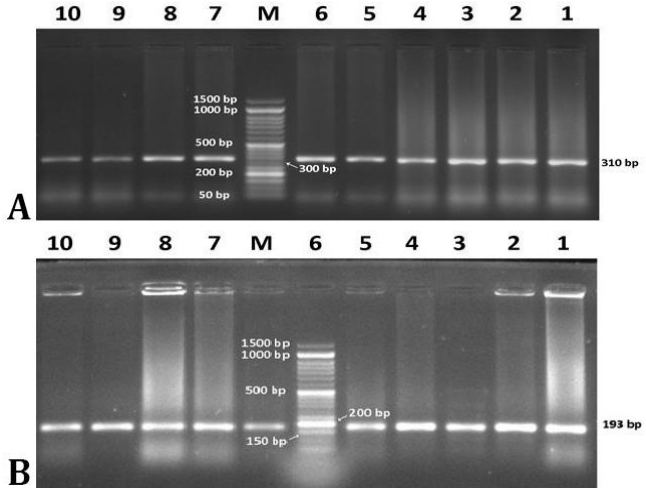

Fig. 3. A) The PCR products of exons 4 of TP53 gene on 1.50% agarose gel. M: DNA Ladder (Jena Biosciences, Jena, Germany); Lanes 1-5: The PCR products in infected samples to OPA; lanes 6-10: PCR products of healthy samples. Both infected and healthy samples showed 310 bpfragment. **B)** The PCR products of exons 9 of TP53 gene on 1.50% agarose gel. M: DNA Ladder (Jena Biosciences); Lanes 1-5: PCR products in infected samples to OPA; Lanes 6-10: PCR products of healthy samples. Both infected and healthy samples showed 193 bpfragment.

As shown in Figure 5, expression of infected samples with C pattern had highest significant differences compared to other groups ($p \leq 0.05$). Also, expression of affected samples with B pattern has significant difference compared to healthy groups ($p \leq 0.05$).

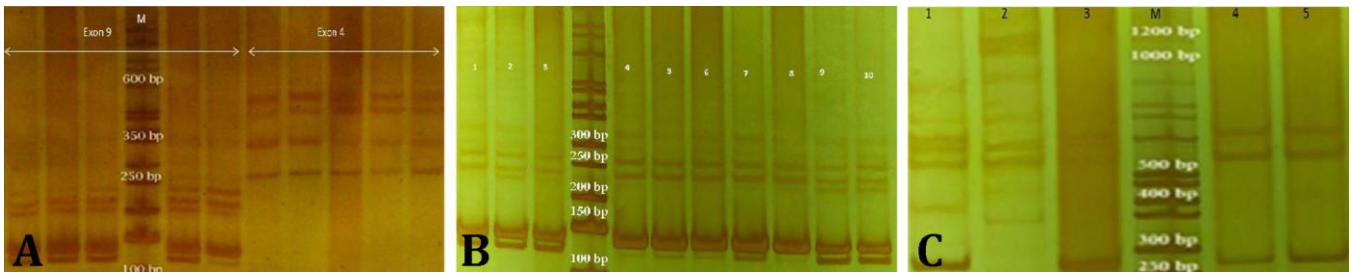

Fig. 4. A) The SSCP patterns of exons 9 and 4 of TP53 gene in the healthy samples. M: DNA Ladder, 50-1500 bp (Jena Biosciences); observed SSCP patterns of exons 9 and 4 in healthy samples. **B**) Some observed patterns of exon 9 of TP53 gene in the infected and healthy samples. Lanes 1-3: SSCP patterns in the healthy samples, Lanes 4-10: SSCP patterns in the infected samples. It was not observed different SSCP patterns among healthy and infected samples. **C)** Some SSCP patterns of exons 4 of TP53 gene in the infected and healthy samples. M: DNA Ladder, 50-1500 bp (Jena Biosciences); Lane 1: A patterns of SSCP; Lane 2: B patterns of SSCP, and Lane 3: C patterns of SSCP; Lanes 4-5: SSCP pattern of healthy samples.

It seems nucleotides mutation were occurred in open reading frame of exon 4, produced an inactive p53 and resulted the tumor (OPA). Inactivation of p53 as tumor suppressor is a frequent event in tumorigenesis.

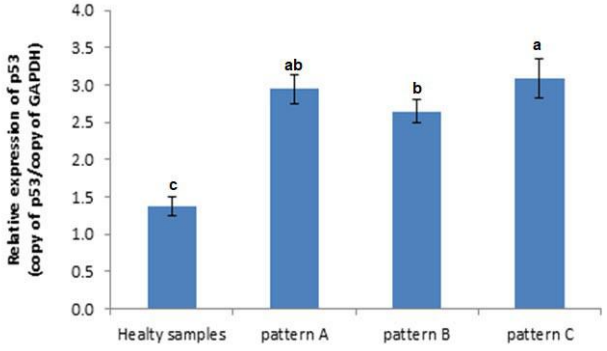

Fig. 5. The TP53 gene relative expression in the observed A, B, and C patterns in the infected samples. Expression of p53 gene in the infected samples content C pattern has highest. All of the infected samples (with A, B, and C patterns) have significant differences compared to control groups ($p \leq 0.05$). The error bars show mean ± SEM. ^{abc} TP53 gene relative expression with different subscript was significantly different at $p < 0.05$.

Discussion

In the present study, observations of macroscopic and microscopic pathology were in line with the specifications mentioned in other studies.[11,12,31-34] Any kind of damages and acquired mutations of the TP53 gene disabled the gene and causes the escape of cancer cells to escape from apoptosis and eventually becomes carcinogenesis. Prevalence of TP53 mutations in more than ten thousand tumor indicates that the tumor origin depends entirely on the possibility of TP53 mutation, for example, the TP53 mutation frequency in lung neoplasia is more than 46.00%, about 34.00% of breast tumors, and in leukemia, is less than 6.00%.[35] Neoplasia with TP53 gene mutations is progressive and human patients with this mutation have a shorter lifespan than patients without TP53 gene mutation.[35] A high proportion of missense mutations of TP53are indicative of selective advantage of mutant type TP53 expressing cells than lacking p53 expressing cells or the cells with wild type p53.[36] Dequiedt *et al*. previously reported that TP53 gene mutation associated with leukemia in a variety species of domestic mammals, including horses, cattle, sheep and goats and, they revealed that this mutation happened in all these species except ovine p53.[26] It was not only reported that the TP53 gene mutation does not occur in testicular tumors[35] but also, it was shown that patients with germ cell tumors (GCT) are mutations in the TP53 gene and p53 tumor suppressors are required for efficient mortality programs.[36] Studies on the association of TP53 gene with OPA disease in sheep did not find any mutation in exons 5 to 8,[26,37] and exon 7 and 8.[38] A previous study reported the mutations in exon and intron 5 and 6 of TP53 gene in pulmonary adenomatous.[39] It was demonstrated that TP53 gene mutation in ovine infected cells of colorectal carcinoma.[40] On the other hand, studies found that tumors associated with amutation in the TP53 genes in other species. Several studies proved the relationship between TP53 gene mutation and cancer in dogs were done on different neoplasms including osteosarcoma,[40] thyroid cancer,[41] mammary tumors,[42] lymphoma[43] and colorectal cancer.[44]

One of the highly accurate, cost-effective, convenient and sensitive techniques for investigation of mutations is SSCP technique. Ovine TP53 gene mutation in the OPA via SSCP technique was previously reported.[26] In the current study, three different single nucleotide mutations were detected among the exons 4 in the infected samples. The TP53 gene encodes several p53 isoforms attributable to alternative promoters (P1 and P2) and exon 4 is within P2 internal promoter of the TP53 gene. This is the first study on exons 4 and 9 of TP53 gene in relation to OPA disease using the SSCP technique. Other studies performed using PCR-SSCP technology on the p53 gene of human lung cancer, including the following: mutations in exon 8 in 74.00% of non-small cell lung cancer cell lines.[9] In humans, mutations in exon 5 to 8 of p53 were reported at a prevalence of 23.90% in lung cancer with smoking and

radon exposure,[45] 78.95% in pulmonary secretions of patientsm[46] and 28.00% in smoking and non-smoking women with lung cancer.[47] In one study, 63.00% of NSCLC tumor specimens had p53 mutation and 26.00% overexpressed HDM2. In this study, p53 inactivation and HDM2 overexpression have been detected at the same time. [48] In the sputum samples of lung cancer patients, rate of p53 mutation was 72.43% that it was detected by PCR-SSCP.[49] In another study, p53 tumor suppressor gene at 13.00% of NSCLC specimens had mutation by PCR-SSCP dectection.[50] In the present study, exon 4 mutations of TP53 gene was observed by PCR-SSCP technique and confirmed by sequencing. In general, three different types of genotype patterns were detected in the infected samples. Moreover, the presence of exon 4 mutations in the infected samples implied the critical and effective role of these mutations in the infection of OPA as a model of lung cancer in human, along with other factors.

Acknowledgments

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Conflict of interest

None.

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