STUDY ON RELATIONSHIP BETWEEN OVINE PULMONARY ADENOCARCINOMA AND P53 TUMOR SUPPRESSOR GENE MUTATION

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ABSTRACT: P53 also known as tumor suppression protein has three main important roles such as growth arrest, apoptosis and DNA repair. Damaged p53 in cells by mutagens (radiation, chemicals or viruses) resulting in cancer. Ovine pulmonary adenomatosis (OPA) also known Jaagsiekte, is a contagious tumour of sheep that caused by a beta retrovirus (jaagsiekte sheep retrovirus: JSRV). The present study was described mutation of p53 gene (enter 7 and 8 exons) on the OPA pathological samples. Fifty pathological samples were collected from Tabriz-Iran slaughterhouse. Eleven out of fifty samples were diagnosed as OPA-affected according to necropsy finding by histopathology and immunohistochemistry. Then, total DNA was extracted from tissues and polymerase chain reaction was run by two pair specific primers. Amplifications products were screened using Sanger sequencing to finding point mutation. The results of this study were showed that there was no mutation through 7 & 8 exons of p53 tumor suppressor gene in ovine pulmonary adenomatosis disease. This is the first study of p53 gene (exons 7 & 8) on pathological samples of Iranian sheep (Gezel). More studies are needed to finding relationship between p53 and ovine pulmonary adenomatosis disease.

Key words: Mutation, P53 tumor suppressor gene, exons 7 & 8, Ovine pulmonary adenocarcinoma

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INTRODUCTION

Tumor suppressor produced a nuclear phosphor protein named P53 which is located on chromosome 11insheep and chromosome 17p13 on human (Kern et al., 1991; Matlashewski et al., 1984). P53 protein appeared in 53-kilodalton (kDa) molecular weight on SDS-PAGE analysis. But, its amino acid residues are only 43.7 kDa. For this reason, P53 protein containing high number of proline residues and proline migrate slowly on SDS-PAGE therefore its molecular weight appear heavier than it actually (Ziemer et al., 1982).
DNA was extracted using Accu Prep® Genomic DNA Extraction Kit. Briefly, lung samples were homogenized with a mortar and pestle. After we placed homogenized samples in a clean 1.5 ml tube, we added 200 µl of Tissue Lysis buffer (TL). For digestion of the proteins we added 20 µl of Proteinase K, mixed by vortex mixer, and incubated at 60°C until the clarity was changed from turbid to clear. Then spun down the tube to remove drops from the inside of the lid, added 200 µl of Binding buffer (GC), mixed by vortex mixer, and incubated at 60°C for 10 min. After this step we added 100 µl of Isopropanol, mixed well by pipetting and briefly spanned down to get the drops clinging under the lid. The lysate was transferred into the upper reservoir of the Binding column tube (fit in a 2 ml tube) without wetting the rim, and centrifuged at 8,000 xg for 1 min. The tubes were opened and the Binding column tubes were transferred to new 2 ml tube for filtration.

When P53 protein binds to DNA, it turns stimulates of translation p21 gene which produce a protein that interacts with cdk2 (cell division-stimulating protein), complexes of p21 with cdk2 inhibit of passing the cell to next stage of cell division. Mutation within p53 result its inability of longer binding to DNA. Consequently the p21 protein is not made available to act as the 'stop signal' for cell division (National Center for Biotechnology Information, 2008)

P53 has managed many mechanisms such as anticancer function, genomic stability, apoptosis, and inhibition of angiogenesis (Ferreira et al., 1999). Mutations affected on ability of the protein to binding to target DNA sequences consequently resulting transcriptional activation prevention of mentioned genes (Brambilla & Brambilla, 1997).

Ovine pulmonary adenocarcinoma (OPA), as well known jaagsiekte (driving sickness), pulmonary adenomatosis or epizootic adenomatosis is a transmissible lung tumor which caused by jaagsiekte sheep retrovirus (JSRV) (Griffiths et al., 2010; Sharp et al., 2003). Sheep are susceptible at all ages; however clinical signs are commonly seen in mature sheep between 2 to 4 years old due to long incubation period (Griffiths et al., 2010; Tustin et al., 1996). Currently, there is no vaccination or treatment for clinical ovine pulmonary adenomatosis which is inevitably fatal (Mostafa et al., 2013).

In the lung, the tumor occurs on secretory epithelial cells and be able to block lung function (Rezazadeh et al., 2012). Mutations often occur in specific region of p53 tumor suppressor gene in some cancers. Generally, the mutation of the P53 gene is one of the key prognostic factors in cell proliferation, DNA reparation and aging (Matlashewski et al., 1984). The p53 gene is a tumor suppressor gene; its activity is against uncontrollable cell growth resulting formation of tumors (Bates et al., 1998).

The TP53 gene can be damaged by cell mutations (chemicals, radiation, or viruses), increasing the likelihood that the cell will begin decontrolled division (Hollstein et al., 1991). The p53 gene is the most frequently mutated gene in human and animal neoplasia with over 10,000 reported mutations (Gruszka-Westwood et al., 2001).

In veterinary most studies were performed on dogs, including thyroid carcinoma (Devilee et al., 1994). Osteosarcoma (Johnson et al., 1998) and breast tumors (Lee et al., 2004; Muto et al., 2000; Wakui et al., 2000) lymphoma (Veldhoen et al., 1998) that in all of these studies, mutations have been identified in tp53. Also, mutations of p53 have been identified horses’ squamous cell carcinoma (Teifke et al., 1996). In cows with BLV, PG53 mutations have been observed (Dequiedt et al., 1995; Ishiguro et al., 1997). In tumors of bovine digestive system changes of p53 genes have been also reported (Scobie et al., 1997). Therefore, it needed to more studies on sheep sp53 gene by considering within exon 7 and 8 to finding accession of these exons and ovine pulmonary adenocarcinoma diseases. Thus, the aim of this study was to investigate the relationship between P53 gene mutations in exons 7, 8 with Ovine pulmonary adenocarcinoma in Gezel breed, by PCR and sequencing of PCR products. In this study, the pfu enzyme was used instead of taq polymerase that its sensitivities too high.

MATERIALS AND METHODS

In this study, a total numbers of 50 samples were collected from Gezel sheep (Iranians breed) in Tabriz-Iran. In this study, a total numbers of 50 samples were collected from Gezel sheep (Iranians breed) in Tabriz-Iran. MATERIALS AND METHODS

Isolation of DNA from lung tissue:

DNA was extract on using Accu Prep® Genomic DNA Extraction Kit. Briefly, lung samples were homogenized (25-50 mg) with a mortar and pestle. After we placed homogenized samples in a clean 1.5 ml tube, we added 200 µl of Tissue Lysis buffer (TL). For digestion of the proteins we added 20 µl of Proteinase K, mixed by vortex mixer, and incubated at 60°C until the clarity was changed from turbid to clear. Then spanned down the tube to remove drops from the inside of the lid, added 200 µl of Binding buffer (GC), mixed by vortex mixer, and incubated at 60°C for 10 min. After this step we added 100 µl of Isopropanol, mixed well by pipetting and briefly spanned down to get the drops clinging under the lid. The lysate carefully was transferred into the upper reservoir of the Binding column tube (fit in a 2 ml tube) without wetting the rim, and centrifuged at 8,000 xg for 1 min. The tubes were opened and the Binding column tubes were transferred to new 2 ml tube for filtration.
After this step we added 500 µl of Washing buffer 1 (W1) without wetting the rim, it centrifuged at 8,000 xg for 1 min. Then the tubes were opened and the solutions were poured from the 2 ml tube into a disposal bottle. Again, carefully we added 500 µl of Washing buffer 2 (W2) without wetting the rim and centrifuged at 8,000 xg for 1 min. For completely remove ethanol we centrifuged once more at ca. 12,000 xg for 1 min. The Binding column tubes were transferred to a new 1.5 ml tube for elution (supplied), then we added 200 µl of Elution buffer on to Binding column tubes, and we waited for at least 1 min at RT (15-25°C) until Elution buffer was completely absorbed into the glass fiber of Binding column tube. In the final step the tubes were centrifuged at 8,000 xg for 1 min to elute. For an improved yield, we eluted the samples twice. Finally, spectrophotometry and agar gel were used to assess the quality and quantity of extracted DNA.

**Concentration Determination of the Extracted DNA**
Concentration and purity of the extracted DNA was measured using a spectrophotometer (Thermo Scientific Nano Drop™ ND-2000, USA) as previously described (Sambrook et al. 1989). The ratio OD_{260}/OD_{280} was used to indicate the purity of the DNAs, where the pure DNA has an OD_{260}/OD_{280} ratio of 1.8 to 2.0. Low ratios could be caused by protein or phenol contamination (Menezes et al., 2002).

DNA samples were also subjected to electrophoresis in an Ethidium Bromide pre-stained 1% (w/v) agarose (CinnaGen, Iran) gel matrix in order to check the DNA quality.

**Primers and Polymerase Chain Reaction (PCR)**
Two pair primers for 7 & 8 exons of ovine p53 tumor suppressor gene was obtained from previously proposed primers by Dequiedt et al (Dequiedt et al., 1995). Sequencing of primers was as below;

**EXONE7:**
- **FORWARD PRIMER:** AGGAACAGGCTCGTGGAGG
- **REVERSE PRIMER:** CGGAGGTGAGGGAAGCTAGC

**EXONE8:**
- **FORWARD PRIMER:** GGTTCACAGGAGAACACAGA
- **REVERSE PRIMER:** CAGAACTGCACCCTCGCTGC

The extracted DNA was used as template for PCR. The PCR reaction mixture contained 2.5 µl of 10x PCR buffer, 0.5 µl dNTP 10mM, 0.5 µl of forward primer 10 µM, 0.5 µl of reverse primer 10 µM, 1.5 µl of MgCl2 25mM, 0.25 µl of pfu DNA polymerase, 1ul (100-1000ng) of sampleDNA and 18.25 µl of dH2O to reach final volume to 25ul.

PCR program for the exon 7 was run as follows: After an initial denaturation at 94°C for 5 min, the PCR mixture was subjected to 35 cycles of 20 s at 94°C, 30s at 59°C, 30s at 72°C, and a final extension step at 72°C for 10 min. PCR program for the exon 8 was run as follows: After an initial denaturation at 94°C for 5 min, the PCR mixture was subjected to 35 cycles of 20 s at 94°C, 30s at 58°C, 30s at 72°C, and a final extension step at 72°C for 10 min. The PCR products were checked on 1.5% agarose gel (Fermentas, Canada).

**Detection and Purification of PCR Products**
Amplified products (approximately 6-7ul) were mixed with 2ul of 6X DNA Loading Dye (Fermentas, Canada) and separated on 1.5 % agarose (Fermentas, Canada). The gel was stained in Ethidium Bromide (Sigma, Canada) solution (0.5µg/ml) for 15 min and the expected size of the PCR products were estimated in relation to 100bp DNA ladder (Fermentas, Canada). Then, total amplified fragments of the gene in expected ranges (sharp and specific bands) were purified from the gel using a gel extraction kit (Gene All, Korea) according to the manufacturer’s instructions. The quality and quantity of the purified DNA were assessed as dsDNA by spectrophotometer and 1% agarose gel as well. Finally, 30ulpurifiedPCR products were sent to sequencing (Bioneer, South Korea).

**RESULTS AND DISCUSSION**
In the present study, from 50 recorded lungs in the slaughterhouse through the pathology eleven samples were diagnosed pulmonary adenomatous which accounted for 22% of all of examined lungs. Slides which were identified to be pulmonary adenomatosis positive were colored by immunohistochemistry method, and then were studied carefully under microscope in six slides the brown color that is caused by antibody-antigen complexes were observed. The ratio between the absorbance A260 /A280 was 1/8 to 2, and there was a strong band with high quality and no elongation, it was defined that DNA extraction did well.
PCR amplification of ovine P53 gene

The exons 7 and 8 of ovine P53 gene were amplified-polymerase chain reaction (PCR) using the specific primers. To improve the amplified efficiency of the exons, the reactions were performed in a Gradient Thermal Cycler (BioRad, Canada) and the products were analyzed by 1% agarose gel with GeneRuler 100 bp Plus DNA Ladder (Fermentas, Canada) to estimate the size of the PCR products. The PCR products were about 210 base pairs (bp) for the exon 7 and 220 base pairs (bp) for the exon 8, respectively, indicating that the primers and amplification condition were specific (Figures 1 and 2).

Sequences Analysis

Sequencing had done by two pair primers (Forward and Reverse) for each exon. Since fragments size is below 300 base pair whole sequences in exons could identified base to base acutely. All results showed that there were no mutations in exons 7 and 8 of p53 tumor suppressor gene. All samples in the exons 7 and 8 of ovine P53 gene had 100% identical alignment with the published sequence of p53 tumor suppressor gene (Accession No: 019468.1). However, our finding showed any mutation within 7 and 8 exons, another study by Olivier et al (2010) shown that high percentage of p53 mutations as single-base substitutions distributed throughout the coding sequence (Olivier et al., 2002). A study by Sanjay Katiyar et al (2000) showed that in hepatocellular carcinoma (HCC) p53 mutations are occur mostly eliminated, penetration, point mutations and more than 90% of p53 mutations in between codons 110 and 307 in the region containing exons 5 to 8 (Katiyar et al., 2000). Results of present study showed no mutation within 7 and 8 exons. Therefore, our finding is not in line with previously studies.
In this study, immunohistochemical results showed that six of the eleven samples have mutations in the p53 gene, but the results of the PCR products had been sequenced for mutations in exons 7 and 8 revealed that no mutation occurred in these samples. The p53 protein is frequently formed and degraded in the healthy animal’s cells. As well as degradation of the p53 protein is related to MDM2 binding. Mutation in p53 proteins often do not stimulate MDM2, as result intent to accumulate at high concentrations in the cells. As a result of immunohistochemical staining p53 mutant becomes brown in color due to very high absorption (Matlashewski et al., 1984) whereas, in normal mode, the stability of p53 was unstable (Bullock et al., 1997).

In this study, we had obtained eleven positive samples (brown color) by immunohistochemistry method but it was not found any mutation. That’s reason may be considered in occurred of the mutant in the others exons of p53 except 7 and 8 exons. In the present study, in the gel electrophoresis of PCR products, there was a non-specific band especially in exon 7. In optimizing of the PCR programs this non-specific band was not removed. It may be due to mismatching of primers to another region of the DNA genomic. We are proposing the exon 7 primers should be to improve in the futures studies. Also, there were extra bands in two samples in exon 8 which was too close to the specific band which Non-specific binding of primers should be consider as a major reason.

In our study, there was one sample that was not amplified by exon8 primers. To solve the problem it was tried to add DNA template in different concentration, used different number of cycles, different annealing temperatures, i.e. but finally the sample was not amplified. That reason may be considered to be mutations in intron because of primers were designed within introns.

A study by Dequiedt et al (1995) showed that Mutations in the p53 tumor-suppressor gene are frequently associated with bovine leukemia virus-induced leukemogenesis in cattle but not in sheep (Dequiedt et al., 1995). This study reports no changes of P53 gene in sheep’s pulmonary adenomatoses that it is compatible with Dequiedt et al which noted (Dequiedt et al., 1995). In another study, Mohammad Zadeh et al (2014) reported point mutation in exon 5 and 6 of p53 tumor-suppressor gene in Iranian sheep related to pulmonary adenomatoses (Mohamadzadeh et al., 2014). However, our study demonstrates that pulmonary adenomatoses disease is not related to mutation exon 7 and 8 of p53 tumor-suppressor gene.

Bucher et al (1996) by a study showed that the frequency of p53 mutations in equine sarcoid might be low (Bucher et al., 1996). Ueda et al (1995) showed no evidence for p53 mutation in hepatocellular carcinoma, except in advanced tumours where a small proportion of cells may have acquired specific base substitutions (Ueda et al., 1995). Also, Hong-Qi Peng et al (1993) reported Mutations of the p53 gene do not occur in Testis Cancer (Peng et al., 1993). Moreover, Heimdal et al (1993) reposed a similar lack of TP53 mutations in a series of 32 patients with bilateral and familial germ cell tumors (GCT) and 2 patients with sporadic GCT in the germline and in addition 15 tumors lacked TP53 mutations (Lowe et al., 1993). Our results indicated that in the investigated samples of pulmonary adenomatoses by positive immunohistochemistry, there are not mutation in exon 7 and 8 ovine p53 gene. This is first study conducted on exon 7 and 8 of ovine p53 gene in Iranian sheep (Gezel breed). More studies are needed to investigate all exons and introns of ovine p53 tumor suppressor gene in the future.

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Competing interests
The authors have no competing interests with this study.

REFERENCES


