P53 MUTATION IN OVINE PULMONARY ADENOMATOSIS

PEYMAN MOHAMMADZADEH, PEJMAN MORTAZAVI AND IRAJ SOHRABI HAGDOOST

abcDepartment of Pathobiology, Faculty of Veterinary Medicine, Tehran Science and Research Branch, Islamic Azad University, Tehran, Iran

ABSTRACT

P53 is a protein which is related to DNA, and has been called tumor suppressor, and is coded in human by the gene TP53. This protein plays a vital role in complex organisms, and regulates cell cycle. It functions as tumor suppressor to prevent occurrence of cancer. P53 is called genome protector or arrowhead of genome protection or head of genome watchers, all of which indicate its protective role in prevention of genome mutations. P53 mutations are the most frequent individual genetic changes in cancers. Acquired mutations in P53 gene are found in all main types of cancer. Among the best points to study mutations of this gene are the lung malignancies. Therefore, in this study, we studied changes of this gene in ovine pulmonary adenomatosis. In this study, 5300 lungs were studied in slaughterhouses of Kurdistan, of which some had white, white-turquoise or nodule-like lesions like those seen in pulmonary adenomatosis (300 samples), which accounted for about 5.6% of all lungs. Of all lungs, samples sized 2*2 cm were isolated and studied under microscope. fifteen cases was positive for ovine pulmonary adenomatosis. Following, positive blocks were colored to study mutation of P53 gene by immunohistochemistry method, of which 6 cases showed mutation. So, frequency of this mutation in positive pulmonary adenomatosis cases was identified to be 40%. Next, positive samples were referred to DNA extraction stage, and then, were sent to GATC Company, Germany, for sequence finding by Sanger’s method using sweep initiator in PCR (two-way reading) method. Based on the results, exon No. 5 was without mutation, intron No. 5 had 27 mutations, exon No. 6 had 6 mutations, and intron No. 6 had 17 mutations, and total number of mutations was 50. Also, the highest number of mutations related to replacement of thymine for adenosine in base No. 201, and in every 6 samples, and followed replacement of by cytosine for adenosine in base No. 223 in 4 samples. This study showed that in ovine pulmonary adenomatosis, like human adenocarcinoma, instances of mutation of P53 gene occur, and these exonic and intronic mutations can cause cancer to occur. In this regard, further studies, particularly those on exons, can be very effective and useful.

KEYWORDS: P53, Mutation, Pulmonary adenomatosis, Sheep

Formation of cancer is a multistage process, which include genetic changes in both genes that create cancer, and genes that suppress cancer. The most common changes related to cancers are those of P53 gene(4). P53 protein, with 387 amino acids is a nucleic phosphoprotein with molecular weight of 53 kilo Dalton, which is encoded by a 20-kilobase gene containing 11 exons and 10 introns, and is placed in human on the short arm of chromosome No. 17 (17p13, 1). In sheep, using FISH technique, locus of P53 gene on chromosome (Q15 19)19 was identified with the same features as human P53(5). It is obvious that any mutation in P53 gene, which disturbs its function, disturbs apoptosis induction in the said cell. Mutations of P53 gene mostly occur as point exclusion, inclusion and mutation. Over 90% of mutations of P53 gene occur in codon 110-307 in a region which contains exons 5-8. In some cancers, mutations often occur in a specific region of P53 gene. Generally, mutation of P53 gene is an important prognostic factor(6). Cancers with mutation in P53 gene are progressive, and the patient has a shorter survival time, compared with patient without P53 gene mutation. Given the importance of mutation of P53 gene in occurrence of various human tumors and in live stocks (Ecke, T.H. et al, 2008), and also considering the role of mutations of P53 gene in occurrence various human adenocarcinoma, in this study, we study presence of mutation; and in case presence mutation is present, we study the locus and the extent of its similarity in different cases in ovine pulmonary adenomatosis and identify sequence of changed patterns(1).

MATERIALS AND METHODS

In this study, 5300 lungs were studies , of which some lacked lesion, some had spread severe lesion.. lesions such as discoloration to white, white-turquoise and nodule- (300 samples) was taken and fixed in 10% buffer solution and reffered to pathology laboratory and slides were prepared, and were studied after they were stained by hematoxylin and eosin method. After confirmation of pulmonary adenomatosis, they were immunostain for P53 by immunohistochemistry method(p53 antibody ?) for the study of mutation of P53gene. In deparaffination stage and in retrieval method, they were placed in EZ machine at pressure of 121
atmospheres and at 100 degrees centigrade for 5 minutes, and having been boiled, they were placed at 80 degrees centigrade for 7 minutes. In this stage, the buffer in which the slide was kept was sodium nitrate based on the used antibody protocol, and its PH was 9. Next, to remove tissue peroxide, pure 37% oxygenated water was diluted with methanol with ratio of 1 to 10. The resultant solution was poured on the tissue so that peroxide would prevent coloring. After this stage, slides were fully washed with water several times, and then, they were washed using distilled water once. Next, for coloring, the primary antibody was applied around the tissue to enclose it using immunohistochemistry specific pen. The antibody used in this stage was diluent antibody, which was diluted with ratio of 1 to 100 and then used. After one hour elapsed, slides were placed in immunohistochemistry specific boxes. Then, immunohistochemistry kit was used. In this stage, the kit with single envision was used, and having been colored by this substance, slides were washed using PBS buffer in three separate vessels for 5 minutes each. In secondary antibody stage, Dako biogenetic kit, called Super N huster, was used. Three drops of this substance were poured on each slide so that they cover the surface of texture fully, and after 20 minutes, slides were washed using PBS buffer in three vessels for 5 minutes each. Next, a number of drops of HRP polymer solution were poured on each slide, and the slides were kept in this condition for 30 minutes. In the meantime, boxes were fully sealed. Having been colored by this substance, the slides were washed using PBS buffer in three vessels for 5 minutes each. Then, for discoloration, DAB substance (concentrated) was distilled using chrome vagen buffer with ratio of 1 cc per one drop, and 2 drops of the resultant substance as applied over each slide. Since P53 protein is an unstable protein, it becomes stable and colorable only if it is somehow mutated. Thus, in fact, slides which have become brown have been mutated. After this stage, slides were fully washed with water several times. Next, they were washed with distilled water once. Then, to create background color, slides were colored by H&E method with very low concentration, and having been washed and dehydrated and dried using alcohol and Xylene, they were studied under microscope. Positive mutated samples, referred to DNA extraction. For DNA extraction, two different methods were used simultaneously. In the first method, to extract DNA, first, 10 paraffinized sections with thickness of 5 micrometer were prepared from paraffinated tissues using standard microtome. However, two-three first sections were discarded, and not used. Microtome machine was washed by Xylene an ethanol before preparing sections, and to prevent contamination with other samples, disposable blades were used. Then, sections were immediately transferred to 1.5 lit centrifuge microtubes, and 1 ml of Xylene was added to each sample, and then they were capped, and firmly shaken for 10 seconds. Next, samples were centrifuged at maximum speed of 12000 rotations per second at room temperature for 2 minutes. Then, the solids on the surface of the centrifuged solution were removed by specific pipettes, and none of pipettes were excluded. Then, 1 ml of 96-100% ethanol was added to each plate, and were fully mixed using vortex. It fully removes ethanol-Xylene extract existing in the sample. Then, samples were centrifuged at maximum speed of 12000 rotations per second at room temperature for 2 minutes. Then, the solids on the surface of the centrifuged solution were removed by specific pipettes, and none of pipettes were excluded. Next, all ethanol remnants were removed by tip of pipette. Then, tubes were uncapped and were incubated at room temperature (15-25°C). Such incubation continued until all residual ethanol evaporated. By this stage, deparaffination was completed. Following, DNA extraction started using IBRC kit. First, 400 micro liter of BLB buffer added to each sample. Then, 20 micro liter of a protease enzyme called K was added to each tube immediately, and was mixed rapidly and fully by vortex (strong mixer), and then, it was incubated at 56°C for 48 hr (in this stage, if RNA-free DNA exist in form of free genome, then, 4 micro liter of RNA substance must be added in amount of 100 milligram per one milliliter, and be incubated at room temperature for 5 minutes). Following, 200 micro liter of pure ethanol was added to each sample, a homogenized solution was obtained by pipetting (at least, 10 times), or by panting vortex (strong mixer) for at least 5 times and for 2 seconds at each time. Then, all the resultant mixture was poured into rotating 2-ml micro-columns using pipette, which were procured already, and they were centrifuged for 1 min at over 9 rpm. Then, these rotating micro-columns were placed in a new 2-ml box, and then, 500 micro liter of BW2 buffer was added to it, and then, it was centrifuged for 1 min at 9000 rpm (this stage was repeated several times). Then, centrifuging was performed for 3 min so that silica membrane (which was embedded on inside of the tube) is dried. Next, thin rotating tubes were placed in a clean 2-ml centrifuge tube, and 50-100 micro liter of EB buffer was directly poured to the center of their silica membrane. Then, they were incubated at room temperature for 5-10
min. Next, they were centrifuged for 1 min at 12000 rpm. In second method, the procedure was as follows: to extract DNA from paraffinized tissue samples, first 10 paraffinized sections with thickness of 5 micrometer were prepared using standard microtome. Then, sections were transferred to 1.5 ml microtubes. However, 2-3 first sections were discarded. Microtome machine was washed by Xylene an ethanol before preparing sections, and to prevent contamination with other samples, disposable blades were used. Then, 600 ml of 1% SDS solution, and 0.1 molar NAOH with acidity of 12.7 and also 10 pieces of chelate 20 (very strong chelating substance for absorption of metals, especially, iron, copper and manganese) (made by Sigma Company, Germany) was added. Then, microtubes were place in water bath (oven) for 45 min at 100°C. After removing the paraffin accumulated on the surface of solution by the head of a sterilized sampler, the liquid was transferred to a 1.5 ml microtube. Having been washed by PBS for 15 min with two iterations, it was transferred to microtube containing the tissue, 500 ml of lysis agent (20 mg/ml K protease), 10 micro liter of Tris-Hel 1M solution, 2 micro liter of 0.5 M EDTA, 100 micro liter of 10% SDS, and 838 ml of distilled water, and was incubated at 50°C for one night, until all parts of the tissue were fully dissolved. Next, 500 ml of phenol-chloroform-isopropanol alcohol were mixed with 25:24:1 ratio using vertex, and added to paraffin-free tissue. Then, it was centrifuged at room temperature at 12000 rpm for 10 min. Then; the liquid accumulated on the surface of solution was removed by head of sample, and transferred to a microtube autoclave. In this stage, a unit volume of chloroform was added to it. Then, it was mixed by vortex and was centrifuged at room temperature at 12000 rpm for 5 min. Next, the liquid over the surface of solution was removed carefully and transferred to a new microtube. Then, 0.1 volume of sodium acetate M4 was incubated for another night at 20°C. Then, deposited DNA was centrifuged at 12000 rpm at 4°C, and liquid accumulated over the surface of solution was discarded. Then, we washed the deposited substance using 75% alcohol once. After centrifuging, the extracted DNA was collected. Finally, having been fully dried under hood, it was solved in 50 micro liter of distilled water. When studying cellular proteins, various types of proteins must be separated. Because biomicromolecules are charged, they may be separated by physical properties such as spatial shape, molecular weight and electric charge by placing them in an electric field. For this purpose, in this stage of this study, we performed electrophoresis on the 2 micro liter of the solution of extracted DNA on agar gel to identify quality, quantity and size of extracted DNA pieces. In this study, the primers proposed by Dequiedt et al in 1995 for study of ovine P53 gene were used. He proposed the following exons for exons 5 and 6.

Exon5:
Forward primer: 5-TCG GTG CTT GTA CAT TCG AC-3
Reverse primer: 5-CAA TCA GGG AGG AAT TAG GG-3
Connection temperature was 57.8°C, and the length of sequence of PCR product was equal to 332 bp.

EXON6:
Forward primer: 5-GGG ACT GTG GAT GGG ACC GG-3
Reverse primer: 5-CC CCA CCT AGG GTG GTC AC-3
Connection temperature was 68°C, and the length of sequence of PCR product was equal to 248 bp.

In this study, preparation of PCR reaction for both primer pairs was prepared as follows with total volume of 40 micro liters: first, all required substances for PCR reaction were poured into a 1.5 milliliter microtube to form master mix. Primary volume of this master mix was formed in a 1.5 microtube based on the sample size. Then, master mix was distributed into PCR-specific 0.2 microtubes based on the sample size. Finally, 30 nanogram of DNA was added to each microtube. After number the microtubes, they were immediately placed in thermocycler and the machine was programmed and started on. After PCR reaction was performed, 10 micro liter of reaction product was subjected to electrophoresis on 1.5% agar gel. After making sure of proliferation of the intended piece, the PCR reaction was purified using PCR product purification kit, called IBRC, the results of which are as follows: and then, then it was sent to GATC Company, Germany, for sequence finding by Sanger’s method using sweep initiator in PCR (two-way reading) method.

PCR program for the initiator pair of exons 5 & 6 was run as follows:

Primary denaturation was performed at 94°C for 5 min. Then, denaturation stage was performed for 20 s at 94°C. Next, this program was followed up with connection of initiator for 30 s at 72°C, followed by
proliferation or expansion of initiator at 72°C for 30 s, which stage was performed in 35 cycles. Then, the final stage, i.e., keeping at 4°C completed. After PCR reaction completed, 10 micro liter of reaction product, was subjected to electrophoresis on 1.5% agar gel. After making sure of proliferation of the intended piece, the rest of PCR reaction was purified using PCR product purification kit called IBRC, and then it was sent to GATC Company, Germany, for sequence finding by Sanger’s method using sweep initiator in PCR (two-way reading) method. PCR product purification protocol using IBRC protocol is as follows: first, PPB buffer in an amount three times as much as the total size of PCR product was added to PCR product, and also isopropanol alcohol with its volume being 30% of the total size of PPB buffer was added to this mixture. Then, we transferred this solution to silica columns and spun it at 14000 rpm for 30 s. Next, the liquid accumulated in the under-tube was discarded, and the column was put back inside the tube. Next, 500 micro liter of PPW buffer was added to the column and was spun for 30 s at 1400 rpm. Next, the liquid accumulated in under-tube was discarded, and column was placed back inside the tube. Next, this stage was repeated once again. Then, the column inside the empties tube was centrifuged for 2 min at 14000 rpm. Finally, the under-tube content was discarded, and silica column was placed inside a clean 1.5 ml tube, and 40 micro liter of EB buffer was added to it. Next, it was incubated at room temperature for 10 min, and was centrifuged at 14000 rpm for 1 min. We then collected the purified PCR product inside 1.5 milliliter tube, and sent it to the said company for sequence finding.

RESULTS

Results of slaughterhouse inspection

In this study, 5300 lungs were studies, of which some lacked lesion, lesion, some had spread severe lesion, and some had lesions such as hydatid cyst. And some others had white, white-turquoise or nodule-like lesions like in pulmonary adenomatosis (300 samples), which accounted for about 5.6% of all of examined lungs. Next, slides which were identified to be pulmonary adenomatosis positive were colored by immunohistochemistry method, and then were studied carefully under microscope. The results of this study are presented in this chart.

Chart 1: level of pulmonary adenomatosis and the number of mutations

The general specifications of slides which were identified to have pulmonary adenomatosis includes: cells which had cubic or columnar epithelium perforate the air ducts, and create finger or pillar perspective and take glandular form (hyperplasia and as result wrinkling into lumen due to low allelic space). Table 1 compares the mutation created in these two exons and their introns.

Figure 1: nodule like lesion show that pulmonary adenomatosis
Table 1: study of number of occurred mutations in samples

<table>
<thead>
<tr>
<th>No.</th>
<th>Exon 5</th>
<th>Intron 5</th>
<th>Exon 6</th>
<th>Intron 6</th>
<th>Total number of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>7</td>
<td>1</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>7</td>
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<tr>
<td>4</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>5</td>
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<tr>
<td>5</td>
<td>0</td>
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<td>2</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Total number of mutations</td>
<td>0</td>
<td>27</td>
<td>6</td>
<td>17</td>
<td>50</td>
</tr>
</tbody>
</table>

As seen, the highest number of mutations occurs in intron 5 and is related to replacement of thymine for adenosine in base No. 201. This mutation has occurred in every six samples. The next highest number of mutations occurs in replacement of cytosine for adenosine in base No. 223, which occurred in 4 samples.

Table 2: study of mutations occurred in exon No. 6

<table>
<thead>
<tr>
<th>Type of mutation</th>
<th>T→G</th>
<th>G→C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Locus of mutation</td>
<td>278</td>
<td>326</td>
</tr>
<tr>
<td>Number</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

As seen, in exon No. 6, replacement of Guanine for thymine, and cytosine for Guanine occurred in base loci No. 278 and 326 in three sample in a fully identical manner.

Table 3: study of mutations occurred in intron No. 6

<table>
<thead>
<tr>
<th>Type of mutation</th>
<th>G→A</th>
<th>G→T</th>
<th>T→G</th>
<th>G→A</th>
<th>T→C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Locus of mutation</td>
<td>399</td>
<td>399</td>
<td>400</td>
<td>406</td>
<td>424</td>
</tr>
<tr>
<td>Number</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>

As seen, the highest number of mutation in intron No. 6 relates to replacement of thymine for cytosine in base No. 424, and these mutations occurred in every six samples, followed by replacement of guanine for thymine in base No. 400, and this mutation occurred in 4 samples.

Figure 2: positive p53 mutation in pulmonary adenomatosis (H&E staining 10x)

Figure 3: positive p53 mutation in pulmonary adenomatosis (immunohistochemistry staining *640)
DISCUSSION

Cancer as disease threatening human life by uncontrollable cell growth can result from various changes in genes controlling cell proliferation, DNA reparation and aging. The tumor suppressor P53 gene is one of the key protectors against such events. In fact, occurrence of cancer is a multistage process which includes genetic changes in genes causing and suppressing cancer. The most common changes seen in relation to cancer are changes related to P53 gene, so that over 1800 acquired mutations of this gene have been identified in human cancers. P53 protein with amino acid 387 is a nucleic phosphoprotein with molecular weight of 53 kilo Dalton, which is encoded by a 20-kilobase gene containing 11 exons and 10 introns, and is placed in human on the short arm of chromosome No. 17 (17p13, 1). In sheep, using FISH technique, locus of P53 gene on chromosome (Q15 19)19 was identified with the same features as human P53. This gene plays a role in regulation of cell cycle. Activation of P53 results in induction or inhibition of over 150 other genes. First, Len and Crawford (1979) described a cell protein which connects to antigen T of virus SV40 (6). The next year, Finali et al showed that the wild type of P53 inhibits cell growth and division. Acquired mutations in P53 gene are found in all major type of human cancer, and almost half of human tumors have a mutation or reduction in P53 gene, which results in its inactivation. Such mutations are seen in different tumors including colorectal cancers (60-65%), breast cancer 30%, and skin cancer 40-60%, human pulmonary adenocarcinoma 30% and in small cells 80%. Mutation of P53 gene can results in deactivation of P53 protein, which can result in tumor expansion and genetic
instability. Deactivation of P53 gene is a key carcinogen event. Presence of P53 MT can provide a selective advantage for evolution of tumor cells rather than supplementing lack of WTP5 activity (14). It has been shown that MTP53 may result in advancement of tumor because expression of some MTP53 proteins in tumor cells with neutral P53 background results in increase of their tumorigenesis ability, whether in vivo or in vitro (39). Although MTP53 proteins are often known as factors which are inactivated in terms copying, some MTP53 proteins which are not able to perform active copying of P63-induced gene can potentially activate copying of genes related to or encouraging survival (9). Some P53 mutants are able to connect to some promoters of target genes of P53 such as P21 WAF/ICF and MDM2, but they are not able to connect to apoptotic gene promoters like bax and PIG3 (18). Also, this category of P53 mutants can cause cell cycle to stop to the same extent that MTP53 can, but they are not capable of inducing apoptosis (25, 28). Inactivation of P53 causes cancer cells evade apoptosis, while they are still stop cell cycle in G2 through CHK1 and CHK2 until breakages of all double strings are repaired DNA (DSBS) (15). This shows that shortage of activity of MTP53 protein only applies to a specific category of genes. Besides, in most gene promoter activated by MTP53, there is no sequence similar to MTP53 consensus sequence to show that transcription of MTP53 may be regulated through response of elements other than those responding to MTP53(40). Changes of P53 gene has been recorded in live stocks. DNA sequences of P53 are very similar in dog, cat, mouse, and cow and human (8, 29). Mutations of P53 have been identified in neoplasms of dog and cat, including thyroid carcinoma (27), osteosarcoma (19, 26, 29) and breast tumors (33,26, 27, 29, 34). Mutations of P53 have been identified horses’ Squamous cell carcinoma; however, important of such mutations is not clear (19). Changes of P53 gen in horses’ sarcoïd have also been reported (24). Unnatural distribution of P53 in cells which show mutated P53 has also been observed in colorectal tumors of dogs (27). In cows with BLV and in dogs with lymphoma, PG53 mutations have been observed (27, 2). In tumors of bovine digestive system and tumors of dog’s bladder, changes of P53 genes have also been reported (29). In a study of dog, in 7 tumors out of 15 different tumors including malignant lymphoma, monocystic leukemia, rhabdomyosarcoma, colon cancer, osteosarcoma, point mutations were excluded and instances of intussusception was recorded (37 ). In another study of six tumors of 30 primary tumors (30%) in dog, including malignant tumors of MPNST, 3 cases of lymphosarcoma, 1 case of zyosarcoma, and 1 case of P53 gene mutation were recorded (22 ). Changes of P53 gene were observed in animal ocular tumors. In a study, 10 tumors of 15 tumors of bovine ocular Squamous cell carcinoma were found to be P53 positive according to immunohistochemistry method. It is said P53 mutation may be at least one of the factors affecting formation of this tumor (6). Post traumatic increases in expression of P53 gene in spindle cell of ocular sarcoma were observed in cat, and spindle cell carcinoma was observed by immunohistochemistry method (4, 16). A high percentage of horse with ophthalma Squamous cell carcinoma also had P53 mutation, and showed changes in expression of P53 (16). Studies on this gene in medicine have largely developed since the early years after gene was discovered so that after molecular studies, the role of P53 mutation was studied in a study titled “role of P53 gene mutation in occurrence of skin squamous cell carcinoma in human“ (13). Mutation and exclusion of P53 tumor suppressor gene are the most common genetic change in lung cancer and especially human pulmonary adenocarcinoma, and are related to progressed cases of the disease (11). Also, occurrence of mutation in P53 gene as a cause of neoplasia in lung was introduced in a study by Alison K. Bauer et al in 2007 titled “role of inflammation in neoplasia in lung". Xang et al (2011) showed in a study titled “molecular and immunological study of human pulmonary adenocarcinoma” showed that P53 gene mutation was seen in 37.9% of the studied population (16). Yuyi et al (2011) demonstrated the important of human papilloma in relation to occurrence of lung cancer in human (4). Demartini et al showed in their study titled “carcinogenicity of retroviruses in ovine pulmonary cancer” that these viruses targeted Clara cells and nomocytes type 2, and suggested that the carcinogenicity of pulmonary adenomatosis always occurred in a multistage process, which started with coating of the first viral protein and cell proliferation, and mutagens associated with this virus were involved in genetic changes including mutation of P53 gene (23). In another study, Walton et al induced lung cancer in mouse by induction of coated protein of the retrovirus that causes jagziekte (11). Mondalis et al also carried out a study titled “comparison of manifestation of P53 gene, cyclooxigenasen, cadherin and β-catenin in ovine colorectal cell carcinoma”, showed role of mutation of this gene in occurrence of this cancer (17). Lerox et al
showed in a study titled “from virus to lung cancer” that the virus that causes jagziekte interferes with target cells through Hyalil 2 and then contributes to induction of cancer with the aid of P3K/akt and kinase routing particles. In this study, the role of P53 was described to be unknown and study of the effect of this mutation on occurrence of this cancer was presented as a suggestion (10). Lio et al showed in a study titled “use of a special PCR method AND ALSO nest measurement PCR method to screen ovine pulmonary adenomatosis” that specific primers, especially those in sequence U7, of viral exon that causes jagziekte were the criterion of screening for this disease (36). Ilhan et al showed in their study on ovine pulmonary Myxoma through immunohistochemistry tests that vimentin played a positive role in occurrence of this disease, but desmin, P53 protein and S100 protein had no such role (37). Finally, Hudachek et al showed in their study on prognosis and also control and retrogression of the disease in lambs infected with ovine lentivirus and also the retrovirus that causes jagziekte that (+)CD3 of immune system played a very important role in natural control and causing retrogression of this disease in both of these infections, and these two disease were highly similar to human varieties of this disease.

Moreover, their studies showed that during the process of the diseases outbreak by this method, no mutation happened in the second tyrosine kinase and P53 did not affect the disease (40).

The above cases indicate that determining the mutations of P53 gene not only is significant in the prognosis of pulmonary tumors in human but also identifying the status of gene P53 in these tumors can play a critical role in selecting treatment protocols (1,26,9,13,19,1). In the present study, several exonic mutations (which are the most important and the main mutations) as well as many intrinsic mutations were respectively observed in exon No. 6 and introns No. 5 and 6 which were of prime significance. In fact, this study reports exonic and intrinsic changes of P53 gene in sheep’s pulmonary adenomatoses. In the same vein, several humanistic studies are done on the changes of the gene; for instance, in a study by Golmohammadi et al (2013) as “studying the characteristics of mutations happened in exons 5 and 6 of P53 gene” in Iran, 21 point mutations in exons 5 and 6 were observed in tumoric specimens from 14 people (%23). Of the amount, %81 and %9.5 mutations were missense. Two mutations existed in intronic area between exons 5 and 6, as well. In 11 mutation samples, the stability of protein and accumulation of protein were detected. There was a relationship between the type of mutation and protein accumulation in exons 5 and 6 of P53 gene. The presence of mutation accompanied an advanced stage of cancer (process, P<0.009). Patients with P53 gene had significance rate less than those with P53 and wild species (P<0.01) (31). Also, in a study as “identifying mutation in exons 5 and 6 of P53 in women suffering from breast cancer in East Azerbaijan Province” by Khoriekhhani et al, 102 tumor specimens were collected from the Azeri race of the area referred to Tabriz hospitals between 2007 and 2009 and their DNA were extracted. Then, P53 gene was proliferated and sequenced from the beginning of exon 5 till the end of exon 6. In this study, 18 changes were observed (%17.64); %7 was related to polymorphism (%6.86), and %11 to mutation (%10.78). The mutations of codon 160 and codon 163 (ATC>AAG) located in exon 5 include %18.2 mutations. Also, %81.8 mutations were observed in exon 6 including codon 193 (CAT>AAT), codon 195 (ATC>TTC), codon 195 (ATC>ACC), codon 198 (GAA>TAA), codon 220 (TAT>TGT), codon 213 (CGA>CTA), and codon 124 (CAT>CGT). No changes were observed in intron 5 and all polymorphisms in exon 6 and nucleotide 13399 were detected. Most mutations identified in this study were of missense type and P53 gene was located at the second connection to DNA. These mutations led to the production of a mutated protein with unstable structure whose capability to connect to DNA has changed (25). Another study as “the polymorphism of codon 72 of P53 gene in patients suffering from nonmelanoma skin cancer in Isfahan” by Mehdi NikbakhtDastjerdi et al was done in test-control form by 20 specimens of Basal cell carcinoma (BCC), 20 specimens of Squamous cell carcinoma (SCC) and 20 test specimens in each case. Different genomes of codon 72 of P53 gene were determined using polymerase chain reaction (PCR). The frequency of Arg/Arg genotypes in BCC specimens was %60 yet %25 in the intact ones. A statistically significant difference was observed between test and control groups in this genotypic group (P=0.048). The frequency of heterozygote Arg/Pro individuals as well as Pro/Pro ones was insignificant in cancer specimens as compared to the natural ones. OR=4.5 in BCC individuals shows that the chance for infection is four and half times higher in individuals with Arg/Arg genotype. X2 test results did not show significant difference between the frequencies of
different genotypes in SCC specimens as compared to the intact ones (14). In another study as “the polymorphism of codon 72 of TP53 gene in patients suffering from endometriosis in Isfahan” by IslamiFarsan et al, the polymorphism was carried out in 90 patients and 90 intact individuals (control group) in Isfahan. Different genotypes of codon 72 of TP53 gene were determined using polymerase chain reaction (PCR). The frequency of Arg/Arg genotype and Pro/Pro genotype was respectively %28.9 and %15.6 in endometriosis specimens and %42.2 and %3.3 in the intact ones. The frequency of heterozygote individuals (Arg/Pro) was %55.6 in endometriosis and %54.4 in the intact group. In comparison, no significant differences were observed between test and control group regarding the frequency of Pro/Pro genotype and the two other genotypes (8).

Another study as “the polymorphism of codon 72 of P53 gene in patients suffering from breast cancer in Isfahan” by Faghani et al was carried out using 51 cancer specimens from invasive channel carcinoma and 51 control ones in Isfahan. Different genomes of codon 72 of P53 gene were determined using polymerase chain reaction (PCR). The frequency of Arg/Arg genotype, Arg/Pro, and Pro/Pro genotype was respectively %43.2, %52.9, and %3.9. the distribution of genotype in cancer group was Arg/Arg in %86.2 specimens, Arg/Pro in %11.8, and Pro/Pro in %2 of them. Statistically significant difference was observed in the distribution of this polymorphism between control and cancer groups (P<0.001) (23). In another study as “P53 gene mutations in hotchkins disease done by AmirhosseinSadrzadehRafi’i et al, the frequency of P53 gene mutation in patients with hotchkinsreferred to Imam Khomeini, Azad and Naft Hospitals between 1991 and 1999 was examined. Microdissection was used for extracting maximum number of Reed Sternberg (RS) cells from the lams provided from patients’ lymph nodes blocks and for carrying out PCR/SSCP of P53 gene (exons 5-8) on DNA product extracted in 40 patients with hotchkins. Results: 6 shifts in SSCP gel, 2 in exon 5, 1 in exon 6, 2 in exon 7, and 1 in exon 8 of five patients (%12.5 out of 40 patients) were observed (21). Another study as “determining mutation in exons 5 and 8 of P53 gene in patients with familial breast cancer” was done by MohammadrezaMirzaii et al using non-radioactive-PCR-SSCP method. There, 32 patients undergone breast biopsy were examined. DNA extraction was carried out by phenol-chloroform method and applied to proliferate exons 5 and 8 using PCR after determining purity degree by spectrophotometry. Using Single Strand Conformation Polymorphism (SSCP) which is the most widely used method for determining P53 gene, single strand products of respective exons were undergone electrophoresis in poly acryl amide gel and dyed using silver nitrate method. Finally, shift changes of resulted bands were compared to the intact specimens and analyzed. Results: after the analysis of SSCP gels and the comparison between the shifts of resulted bands and control specimen, 2 mutations in exon 5 and 4 in exon 8 were determined (36). In another study “exploring the promoter mutations and exons 2-4 and 9-11 of P53 gene using PCR-SSCP method in patients with gastric cancer in Chaharmahal-o-Bakhtiar Province” done by JavadSfariChaleshtari et al, promoter mutations and exons 2-4 and 9-11 of P53 gene were studied in gastric biopsy specimens in 38 patients win the provine. DNA extraction was done using phenol chloroform standard method and molecular tests were carried out on respective sequences using PCR-SSCP method. In results, no evidences were observed regarding the occurrence of mutation in promoter and exons 2-4 and 9-11 of P53 gene (4). In another study as “molecular examination of P53 genes in patients with breast cancer using non-radioactive PCR-SSCP method” by KatayounE’temadi et al, P53 gene mutations were examined in exons 6 and 7 using PCR-SSCP method and the mutation amount was compared based on hystopathological factors of tumor and the existence of familial history and patients’ age and using Fisher exact statistical test. 24 patients with basic breast cancer were chosen. Specimens were examined for DNA extraction after pathological diagnosis and the review of familial history for cancer.

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