Immunohistochemical detection of \textit{P53} and \textit{Mdm2} and its correlation with histological grading system in ovine pulmonary adenocarcinoma

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\textbf{Abstract}

Ovine pulmonary adenocarcinoma (OPA) is a cancer disease in sheep caused by Jaagsiekte sheep retrovirus (JSRV). The retrovirus is distinctive among viruses for inducing carcinogenesis of lung epithelial cells and cause a lung adenocarcinoma. OPA has numerous characters same as human lung adenocarcinoma, involving a similar histological organization and motivation of most cell signaling pathways. \textit{P53} pathway is frequently changed in human lung adenocarcinoma, in specific due to the increase expression of \textit{Mdm2} and it is the main regulator of \textit{P53}. Here, we have a go at something new to confirm the possible expression of \textit{P53} and \textit{Mdm2} in OPA as a translational animal model for human lung adenocarcinoma, and to identify the correlation between \textit{P53} and \textit{Mdm2} expression. 1645 of lung samples from different breeds were macroscopically tested. OPA was recognized in 21 samples and further assessed by histology and immunohistochemistry. Histologically, proliferative cancer foci were distributed and contained of cuboidal or columnar cells and arising papillar to acinar patterns. The nuclear expression of \textit{P53} and \textit{Mdm2} was detected in 90\% and 95\% respectively in the cancer epithelial cells of OPA respectively. Detectable immunoreactivity for \textit{P53} was detected in 6 out of 7 grade I, 7 out of 8 grade II, and 6 out of 6 grade III cancers. In reverse with \textit{P53}, \textit{Mdm2} was detected in 18 cases with moderate and high expression. In addition, there was statistically relationship between both protein expressions. Our findings suggested that overexpression of \textit{Mdm2} plays an essential part in OPA carcinogenesis and is dependable on the grading system, and its overexpression can be convinced by \textit{P53} expression.

\textbf{Introduction}

Ovine pulmonary adenocarcinoma (OPA) is a chronic infectious sheep lung cancer caused by jaagsiekte retrovirus (JSRV)\textsuperscript{(1)}. OPA remains a substantial economic challenges for farmers and represents an important model for human lung adenocarcinoma. JSRV has been noticed in both human and sheep pulmonary adenocarcinoma. In addition, there may be similarities in the motivation of oncogenic signalling pathways\textsuperscript{(2)}. \textit{P53}/\textit{Mdm2} pathway has been considered as one of the important route in the progression and development of human lung adenocarcinoma\textsuperscript{(3)}. Therefore, there is a need to investigate the possible expression of these two proteins in OPA. As a result of many stressors, such as viral infection, modifications of the \textit{P53} gene have been recognized in many types of human cancer, and progress of nearly most of human cancer is linked to mutations that lead to deactivation of this gene\textsuperscript{(4)}. Actually, mutations of \textit{P53} gene permits cancer cells to get away from apoptosis and key event in carcinogenesis\textsuperscript{(5,6)}, lead to overexpression of \textit{P53} protein\textsuperscript{(7,8)}. The expression of this protein contributes in apoptosis of cells with damage of DNA and also shows an important part in cell cycle switch leading to uncontrolled cell growth\textsuperscript{(9)}. The transcription factor of \textit{P53} gene can stimulate the transcription of many downstream genes which regularly facilitates their biological functions.
One of the important downstream genes is protooncogene mouse double minute 2 (MDM2) (11). Mdm2 is highly expressed in most types of cancer and plays an essential role in cancer progression and development (12). Unnecessary Mdm2 overexpression can lead to constitutive suppression of P53 and encourage unrestrained cell cycling (13). Interaction among Mdm2 and P53 can suppress the transcriptional function of P53 and lead to elimination and mutation of P53 during proteolysis (13). Several studies show that overexpression of mutant P53 may stop Mdm2 degradation, contributing to the increasing of Mdm2 in most human cancer cells (14).

Here for the first time, we undertaken to analysis the frequency of P53 and Mdm2 expression in OPA as well as to identify the relationship between expression of P53 and Mdm2 using histological grading system and immunohistochemistry.

**Materials and methods**

**Sample selection**

The records of lung lesions in various breeds sheep (1645 samples) that were collected from abattoirs in Nineveh, Iraq between November, 2019 to May, 2020 were reviewed. A total of 26 lesions were selected as lung tumors, twenty-one cases of those were of OPA. The samples were obtained for histology and immunohistochemistry analysis. The positive control was used from a paraffin-embedded breast carcinoma that was already investigated to have a P53 and Mdm2 expression.

**Histopathologic analysis**

All tissue samples were fixed in 10% buffered formalin, and were routinely processed and embedded in paraffin. The sections were stained with Haematoxylin and eosin, Masson’s trichrome and PAS stain. The histological classification of OPA was based on the WHO classification for human pulmonary adenocarcinoma (15). The histological parameters of OPA were classified into two main types classic and atypical (16). Each type was categorized into three grades depending on the mitotic figure, nuclear changes and shape of the cancer cells (17). The degree of differentiation was graded as Grade I, >90% (well-differentiated), Grade II, 50-90% (moderately differentiated), and Grade III, <50% (poorly differentiated). Every field was graded consistent with the grade and next the overall Grade was divided by ten.

**Immunohistochemistry analysis**

Immunohistochemistry was achieved by using the avidin-biotin immunoperoxidase technique. The adhesive slides were dewaxed and rehydrated. Endogenous peroxidase was blocked in 3% hydrogen peroxide-methanol solution for 30 min. Then, the slides were washed in phosphate buffered saline (PBS) (PH 7), the nonspecific proteins were blocked by blocking solution for 1 hour at room temperature. The slides were incubated with primary antibodies (P53 Rabbit Polyclonal, dilution 1:100, Wuhan Fine Biotech, China) and (Mdm2 Rabbit Polyclonal, dilution 1:100, Wuhan Fine Biotech, China) for overnight at 4°C. Once washing with PBS, the slides were incubated with poly-HPVoat Anti-Rabbit IgG (dilution 1:100, Wuhan Fine Biotech, China) for 1 hour at 37°C. After another PBS washing, the reaction was amplified with an avidin-biotin complex. The slides were counterstained with haematoxylin, rinsed in distal water, dehydrated and coverslipped. For positive control, slides of breast cancer were used for both antibodies. For negative control, Non-immune serum was replaced for the primary antibodies and the rest of the steps was same. Using Image J program, P53 and Mdm2 staining was evaluated by find out the density of positive nuclei. P53 and Mdm2 expression was measured using a 3-point Grading system, as: 0% (Grade -), 1-10% low (Grade +), 10-50% moderate (Grade ++) and >50% high (Grade ++++) (18). Every field was graded consistent with the Grade and then the overall Grade was divided by ten.

**Results**

In 21 of the 1645 tested lung sample, cancer masses were recorded on all lobes of lung. However, the most affected part was situated in the dorsal area of the caudal lobe of lung. The cancer masses were of many nodules with white appearance (Figure 1). These lung nodules were firm structure with irregular edges ranging from 3-5 cm diameter. Around the large nodules, there was an atelectatic pink edge. After cutting the nodules, frothy fluid was observed mainly in the ventral part of the lung (Figure 1a and b).

Histologically, in all 21 samples, cancer foci of OPA surrounded and divided by fibrous connective tissue (Figure 1c). These foci contained of columnar or cuboidal cells which lining the affected alveoli and making papillary to acinar patterns that protruded into the lumen of alveoli, bronchi and bronchioles. The cancer cells were infrequently vacuolar, including large nucleus with highly mitotic figure index and poorly differentiation. In addition, around the cancer foci, moderate accumulation of hypertrophied macrophages (Figure 1d). Using Masson’s trichrome stain, large amounts of collagen fibers were observed around the neoplastic growths (Figure 1e). Positive reaction with Schiff’s reagent was detected in the cancer cells in PAS staining techniques (Figure 1f). Based on the WHO classification for human pulmonary adenocarcinoma, 7 samples of OPA (33%) were considered as grade I, 7 (38%) as grade II and 6 (29%) as grade III.

The detailed of immunohistochemical results of P53 and Mdm2 expression depending on their grading system is shown in Table 1. The nuclear expression of P53 was detected in 19 out of 21 (90%) in the cancer epithelial cells of OPA. Three of the 7 grade I carcinoma, exhibited strong P53, two showed moderate P53 and two showed either mild or negative nuclear expression.
Four of the 8 grade II carcinoma, exhibited clear P53, two showed moderate P53 and two showed either mild or negative nuclear expression. Four of the 6 grade III carcinoma, exhibited clear P53 (Figure 2), two showed moderate P53 and naught showed either mild or no nuclear expression. All results were compared with human breast cancer (positive control) (Figure 2) and replacing the P53 antibody with non-immune serum (negative control) (Figure 2). To study a relationship between P53 and Mdm2 expression, samples with strong to moderate nuclear immunoreactivity for P53 and Mdm2 were considered as positive. sixteen of 17 Mdm2 positive carcinoma were P53 positive, and zero was Mdm2 negative. Four of 1 Mdm2 positive carcinoma was P53 negative, and 3 was Mdm2 negative (Table 2). There was statistically relationship between both protein expressions.

Figure 1. Gross lesion and histological appearance of OPV. a. Cancer mass in the dorsal area of the caudal lobe of lung. b. Firm structure nodules with irregular edges ranging from 3-5 cm in diameter. c. Cancer foci surrounded by fibrous tissue (red arrow), which contains cancer cells (black arrow), and forming papillary patterns (blue arrow). H&E. 200µm d. Cancer foci contained of cuboidal or columnar cells (black arrow), with poorly differentiated cells (red arrow), and infiltration of hypertrophied macrophages around the cancer foci (blue arrow). H&E. 50 µm. e. Cancer foci contained of cuboidal or columnar cells (red arrow), with poorly differentiated cells (black arrow) and dense deposition of collagen fibers that stained green with Masson’s trichrome stain (green arrow). Masson’s trichrome stain. 50µm. f. Columnar cancer cells (black arrow) with positive reaction with Schiff’s reagent in PAS staining protocol (red arrow). PAS stain. 50µm.

Figure 2: Immunohistochemical staining for P53 protein. a. Marked nuclear expression of P53 was detected in the cancer epithelial cells of OPA (grade III). IHC. 200µm. b. Marked nuclear expression of P53 was detected in the cancer epithelial cells of OPA (grade III). IHC. 50µm. c. A human breast cancer, which expresses P53 protein, is used as the positive control. IHC. 200µm. d. A human breast cancer, which expresses P53 protein, is used as the positive control. IHC. 50µm. e. Negative control excluded the P53 primary antibody but involved all other steps with non-immune serum. IHC. 200µm. f. Negative control excluded the P53 primary antibody but involved all other steps with non-immune serum. IHC. 50µm.
Table 1: Immunohistochemical findings of \( P53 \) and \( Mdm2 \) in ovine pulmonary adenocarcinoma

<table>
<thead>
<tr>
<th>Grade</th>
<th>( n )</th>
<th>( P53 )</th>
<th>( MDM2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Grade I</td>
<td>7</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Grade II</td>
<td>8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Grade III</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
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Table 2: Correlation between of \( P53 \) and \( Mdm2 \) expression in ovine pulmonary adenocarcinoma

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>( Mdm2 ) (+)</th>
<th>MDM2(-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( P53 ) (+)</td>
<td>17 (81%)</td>
<td>17</td>
</tr>
<tr>
<td>( P53 ) (-)</td>
<td>4 (19%)</td>
<td>1</td>
</tr>
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(-) = low or no expression; (+) = moderate and high expression. \( P < 0.05 \) is considered statistically significant using chi-squared.

Discussion

OPA is an infectious lung cancer that affects approximately all sheep-rearing nations across the world (19). It has been reported variable occurrence of OPA in different areas, determined by managing system, hygiene, and age (20). In this study, OPA was diagnosed in 1.27% of all samples collected from abattoirs in Nineveh, Iraq between 2019-2020. Definitely, only healthy sheep is slaughtered indicates that the true predominance of OPA could possibly be much higher and many cases are likely to persist undiagnosed. The morphological features of OPA has been classified into two types classical and atypical (21). In the current study, the classical form is mainly identifying and the lesion affect all lobes and can be either diffuse or nodular type, exhibiting a white moist appearance on the cross section. In addition, some cases were diagnostic as atypical form which was dry white hard nodules.

The histological features of OPA consist of many proliferated foci of cuboidal or columnar cancer cells rising in the alveoli or from the wall of bronchioles. These foci have a papillary or acinar manifestation and in some samples often have areas of necrosis at the middle. Furthermore, it was distinguished that increasing cancer cells mainly initiate from type-2 pneumocytes (22). The cancer cells grow in the normal lung and produce loss of lung function and increase secretion of the fluid (23). The primary illustration of OPA indicated that the disease is not aggressive. Nevertheless, local metastases are found in 10% of infected sheep, approving that OPA is a cancer disease (24). Furthermore, OPA has some of the clinical and histological types of the malignant human lung adenocarcinoma. While there is no fit model for human cancer disease, it was shown that adequate relationships among OPA and human lung adenocarcinoma to support that understanding the molecular biology of OPA could benefit to know some features of human lung oncogenesis (2).

\( Mdm2 \) is an oncoprotein that down regulate the \( P53 \) tumour-suppressor protein. These two proteins promote
proliferation and cell survival throughout a many mechanisms involving activating cell growth and division and inhibiting apoptosis (25). Mdm2 expression has been identified in many human cancers including pulmonary adenocarcinoma, and these expressions have a function in carcinogenesis by suppression of P53 normal function (26). One of the most interesting issues about the biology of OPA concerns the way by which the JSRV-persued lung epithelial cells to transform to cancer cells.

For retroviruses, carcinogenesis usually follows after the high levels of viral infection in the target tissues (27). Therefore, it has been doubtful if JSRV persuades OPA by stimulation of Mdm2 and suppression of P53 function. This occurrence is now measured to be relatively frequent. In the OPA samples considered here, the P53 protein was noticed in the nucleus of epithelial lung cancer cells in 90% of lung samples. It has been indicated that high nuclear P53 immunoreaction in most cancers indicates histologic malignancy (28). The high expression of P53 seemed to be positively associated with increasing grading system. The level of mutated P53 was highly expressed in grade III samples compare to grade I. These results propose that prolonged life span of mutated P53 produces the accumulation in the nucleus of lung cancer epithelial cells, permitting it to be noticed by immunohistochemistry (29). In addition, in this study, the overexpression of Mdm2 is amplified with the increasing histological grading system. The level of nuclear expression of Mdm2 is higher in grade III compare to grade I samples. These results propose that Mdm2 plays a significant part in carcinogenesis of cancer epithelial cells. It has been shown that overexpression of Mdm2 in human lung adenocarcinoma have a significant part in carcinogenesis throughout suppression the function of P53 tumour-suppressor protein (30). In our study, the expression of P53 and Mdm2 in OPA was compared with the positive over reaction of both proteins in the breast cancer samples (31). Both P53 and Mdm2 proteins were overexpressed in most of the OPA samples in this study, and the overexpression were positively correlated which may have prognostic value.

**Conclusion**

Our study confirmed that overexpression of Mdm2 plays a significant role in the progressing of OPA, and is consistent with histological grading system, and it expression can be increased with mutant P53 overexpression in the ovine epithelial cancer cells.

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**Conflicting of interests**

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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الكشف الكيميائي التصنيفي المناعي عن Mdm2 و P53 وارتباطه بنظام التصنيف النوعي في سرطان الغدد الرئوية في الأغنام

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الخلاصة

سرطان الغدد الرئوية في الأغنام هو مرض سرطاني يصيب الأغنام وينتج عن الفيروس الارتجاعي للاغنام. يعتبر الفيروس الارتجاعي من عائلة الفيروسان. يلعب دوراً أساسياً في توليد السرطان في الغدد الرئوية. هذا السرطان يتميز بانتشاره في الغدد الرئوية وانتشاره في الأنسجة المجاورة.


تم الكشف عن P53 في سرطان الغدد الرئوية في الأغنام. P53 هو البروتين الذي يعمل على تحفيز الكشف عن سرطان الغدد الرئوية في الأغنام. P53 هو البروتين الذي يتحكم في التحكم في النمو الخلايا ويعمل على تحفيز الكشف عن سرطان الغدد الرئوية في الأغنام.

لدينا دراسة جديدة لتأكيد التعبير المحتمل عن P53 في سرطان الغدد الرئوية في الأغنام. P53 هو البروتين الذي يعمل على تحفيز الكشف عن سرطان الغدد الرئوية في الأغنام. P53 هو البروتين الذي يتحكم في التحكم في النمو الخلايا ويعمل على تحفيز الكشف عن سرطان الغدد الرئوية في الأغنام.

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