TWO-DIMENSIONAL ELECTROPHORETIC ANALYSIS OF POLYPEPTIDES EXPRESSED BY NORMAL AND OVINE PULMONARY ADENOCARCINOMA AFFECTED LUNG TISSUES

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Abstract

Frozen lung tissue sections from 2 healthy and 2 adenocarcinoma affected sheep were lysed in appropriate buffer. The two-dimensional (2D) electrophoresis of the protein lysates was performed. The resulting gels were visualised by silver and Coomassie Blue staining, then scanned and analysed using appropriate software. There was one spot present on the image obtained from the analysis of healthy tissue and no spot was found on cancer 2D gel image. The spot was excised and analysed using mass spectrometry. As a result, cytosolic NADP-isocitrate dehydrogenase was identified. In addition, several other protein spots of different intensity in neoplastic tissues, as compared with healthy ones, were found. The last finding reflects changes in protein expression in neoplastic and healthy tissues. These preliminary results can serve as the basis for more detailed investigations of the neoplastic tissue proteome, e.g., isoelectric focusing in narrow pH range and analysis of correlation between tissue and serum protein profiles. The analysis of serum proteins from affected sheep can reveal markers of neoplastic process and help in preclinical diagnosis of ovine pulmonary adenocarcinoma.

Key words: sheep, ovine pulmonary adenocarcinoma, two-dimensional electrophoresis, proteome.

Ovine pulmonary adenomatosis (ovine pulmonary adenocarcinoma, jaagsiekte, OPA) is a disease caused by jaagsiekte sheep retrovirus (JSRV) – a member of the genus Betaretrovirus. The virus induces neoplastic transformation of secretory epithelial cells of sheep lung: alveolar type II pneumocytes and Clara cells.

The disease occurs in most of the sheep rearing countries. It has long incubation period and clinical signs are those of progressive respiratory illness. During the final stage of the disease, animals exude a frothy white fluid from the nose. The fluid accumulates in the respiratory tract and the disease has irreversibly fatal outcome (23). The infection is persistent and there is no immune response, due to the presence of similar endogenous retroviruses. At present, there are no approved tests for jaagsiekte, especially before clinical signs occur, and only histological examination of the lungs is a way to diagnose the disease (4). OPA is clinically and histologically similar to human bronchioalveolar cancer (BAC). The possibility of experimental induction of the tumour in animals makes it a good model for the study of oncogenesis and target therapy of lung adenocarcinoma (17).

The two-dimensional electrophoresis (2D electrophoresis) is a basic tool in proteomic studies. It allows for resolving big amount of proteins in one gel, on the basis of their isoelectric points and molecular masses (6). These advantages make it a useful tool in cancer biomarker discovery. Together with mass spectrometry, it may allow for the identification of the protein changes occurring during the transformation of a healthy cell into a neoplastic cell. Proteome refers to all of the proteins in a cell, tissue, or organism and “clinical proteomics” describes the study of proteomes in the health and disease (20). Comparison of neoplastic tissue proteome with healthy tissue proteome may give information about protein expression differences between them. If tumour-related proteins occur in blood or other accessible body fluids it may lead to the possibility of diagnosing cancer at an early stage by collecting such fluids and testing them for the presence of those biomarkers. When detected in high amounts in blood, they can be suggestive of tumour activity. Biomarkers found in blood and other fluids might also be valuable for monitoring the response to cancer during treatment or detecting the recurrence of tumours after treatment (19).

The present study was intended to examine the proteomes of OPA affected and unaffected sheep lung tissues for discovering protein expression changes.
Material and Methods

Tissue samples. Two frozen samples of ovine pulmonary adenocarcinoma obtained from 2 adenomatosis affected sheep were kindly provided by Dr M. De Las Heras, University of Zaragoza, Spain. Two lung samples obtained from non-affected sheep were collected post mortem in an abattoir. All the samples were stored in a deep freezer (–70°C) until use.

Sample preparation. Frozen blocks (200 mg wet weight) of each tissue were ground in a mortar containing liquid nitrogen to a frozen powder, then added to appropriate amount (2 ml) of sample lysis buffer containing 9 M urea, 4% CHAPS, 50 mM dithiothreitol (DTT), 0.2% Bio-Lyte 3/10 ampholytes (Bio–Rad), and 0.001% protease inhibitor cocktail. The samples mixed with buffer were incubated for 10 min in ice, homogenised with a sonicator (3 times for 15 s with 1 min breaks for cooling) and centrifuged at 25 000 rcf (relative centrifugal force) for 1 h at 4°C. The resulting supernatant was kept at -70°C until use. The protein content was measured using Bradford method (2).

2D electrophoresis. (a) First dimension (Isoelectric focusing - IEF). Appropriate volumes of each sample lystate were diluted in rehydration buffer (9 M urea, 3% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10 ampholytes) to final volumes of 300 µl. The protein load for silver staining was 80 µg, for Coomassie staining - 300 µg. Three hundred microlitres of each sample was loaded into channels of isoelectric focusing tray. Polyacrylamide gels with immobilised pH gradients 17 cm long, pH 3-10 (Bio-Rad) were placed gel side down onto loaded samples and overlaid with mineral oil. IEF focusing tray was placed in the Protean IEF Cell (Bio-Rad). Rehydration under current (active rehydration) was performed for 15 h in 18°C at 50 V. Prior to isoelectric focusing, paper wicks wetted with deionised water were placed between IPG strips and electrodes. The electrophoresis ran in three steps in the following conditions: the first step at 250 V (15 min), the second step at 4000 V (2 h), the third step for 60 000 volt-hours; the current was maintained at 50 µA/IPG strip; temperature - 20°C.

(b) Second dimension electrophoresis using SDS-PAGE. The strips were transferred into another plastic tray and equilibrated with a buffer containing 0.375 M Tris-HCl, pH 8.8, 6 M urea, 20% glycerol, 2% SDS, and 2% (w/v) dithiothreitol, for 15 min as the first step and with 2.5% (w/v) iodoacetamide instead of dithiothreitol for another 15 min as the second step. For SDS-PAGE separating gels (12% of monomer concentration) and stacking gels (4% of monomer concentration) were prepared according to Laemmli buffer system. The equilibrated IPG strips were laid on top of each polyacrylamide gel and were covered with 0.5% agarose solution containing bromophenol blue. The electrophoresis was performed in PROTEAN II XL cell (Bio-Rad), in TGS running buffer (25 mMTris, 192 mM glycine 0.1% SDS), under constant current conditions: 16 mA/gel for 30 min, then 25 mA/gel until the bromophenol blue reached the bottom of the gel.

Gel staining. For silver staining, each gel was incubated in a fixing solution (10% acetic acid and 40% ethanol) two times for 1 h, then sensitised with the mixture of 75 ml of ethanol, 10 ml of 5% Na-thiosulphate, 17 g of Na-acetate, and H2O to a final volume of 250 ml. After washing with water 5 times for 5 min, the gels were treated with cooled silver nitrate solution (0.625g AgNO3 in 250 ml of H2O) for 1 h, then washed with water 3 times and incubated for 3-5 min in developing solution (6.25 g of Na-carbonate, 115 µl of formaldehyde, 7 µl of 5% Na-thiosulphate solution, and H2O to a final volume of 250 ml for each gel). Developing was stopped with EDTA solution (3.25 mg of EDTA in 250 ml of H2O) for 45 min. The gels were washed then with water 3 times for 5 min. The Milli-Q water only was used for washing and preparing the solutions in this method. 2D electrophoresis of each sample was repeated using the same conditions. The resulting gels were scanned for further analysis of their images in PDQuest software (Bio-Rad). Gel images of tumour and non-tumour samples were compared one to another. To confirm significant differences in protein expression, 2D electrophoresis with higher amounts of proteins loaded (300 µg/gel) was performed. Staining with Colloidal Coomassie Blue G-250, as a method more suitable for protein identification with mass spectrometry, was chosen. The gels were fixed for at least 3 h in 50% ethanol/3% phosphoric acid, washed 3 x 20 min. in water, pre-incubated for 1 h in 34% methanol/3% phosphoric acid/17%(w/v) ammonium sulphate solution, then Coomassie Blue G-250 was added to the solution (0.35g/1 L of solution ) and gels were stained for 4-5 d. Afterwards, they were washed 3 times in water to remove the background staining.

Results

The 2D gel images were imported in a 2D gel image analysis programme on a set of six gels per sample. PDQuest 7.2.0 was used to locate and quantify protein spots and to match spots through the gels. The main difference between protein profiles obtained from normal lung tissue and cancerous tissue was the intensity of several spots, correlated with the amount of particular proteins and evidenced on 2D gel images. This finding reflects different levels of protein expression in neoplastic and healthy tissues (Fig.1).

Analysis of the protein expression using 3D simulation of the protein spots allowed for an objective view for the comparison of spot intensity between the images. Each protein spot could be presented in 3D views by its relative amount and distribution. The 3D peak of the protein spot was generated based on the pixel versus area data (Fig. 2).

Several horizontal rows of spots were seen on the gels. The rows of protein spots, which typically had similar molecular sizes but slightly different isoelectrical points, probably were due to posttranslational modifications that change the protein charge. Because of big amount of spots and high abundance of some
proteins, it was difficult to find significant differences between cancer and normal tissue; however, there was one spot present on the image obtained from the analysis of non-tumour tissue and not found on cancer 2D gel image (Fig. 3).

Gel piece containing the spot was manually excised and analysed using mass spectrometry. As a result, cytosolic NADP-isocitrate dehydrogenase was identified (accession No. Q6XUZ5, molecular weight 46.783 Da, according to SWISS-PROT database).

**Fig. 1.** Images of two-dimensional gels stained with silver nitrate: gels 1 and 2 show electrophoretic profiles of ovine pulmonary adenocarcinoma, gels 3 and 4 – profiles of non-tumour sheep lungs.
Fig. 2. 3D simulation of protein spots provided by PDQuest software. Areas in boxes seen on gel images correspond with diagrams below them. The peak area showed the distribution of the protein spot in the gel, whereas the volume correlated to the protein amount.

Fig. 3. 2D gel images compared using PDQuest software. The spot marked as ‘A’ is present in gel obtained from non-tumour tissue, absent in matched gel of tumour proteins profile. The protein obtained from the first gel was found to be a cytosolic NADP-isocitrate dehydrogenase. The ‘B’ and ‘C’ areas are examples of intensity differences of matched spots in the samples.

Discussion

The proteomic approach shows great potential to be a powerful tool for the identification of proteins differentially expressed in ovine pulmonary adenocarcinoma affected lung. The introduction of immobilised pH gradients and advanced bioinformatics has greatly improved the reproducibility and comparability between gels, although 2D electrophoresis is still not a routine method for a clinical laboratory due to the high labour intensity (9). However, for research purposes, 2D electrophoresis is still extremely valuable. Proteomic studies on lung cancer were performed so far only in human lung oncology (7). There are no findings showing protein profiles of ovine pulmonary adenocarcinoma.

In this study, one significant difference was found in matched proteomes – the presence of cytosolic NADP-isocitrate dehydrogenase in non-tumour tissue samples that was not found in the cancer sample. The protein belongs to the group of enzymes significant for isocitrate metabolism by its function in catalysing the oxidative decarboxylation of isocitrate to α-ketoglutarate. According to Kil et al. (12) it may play an important regulatory role in cellular defence against oxidative stress. It also seems to be a major NADPH producer required for fat and cholesterol synthesis (14). Liu et al. (15, 16) found that the expression of that
enzyme in bovine mammary gland is modulated by prolactin and metabolic effectors. In lung tissue, the role of its expression is still not defined. In human lung oncology, there were several reports on differences in protein expression (2, 8, 22). The authors used other pH ranges than 3-10 in IEF and analysed the protein spots with mass spectrometry showing differences in their intensity as significant markers; however, there was no report on cytosolic NADP+-isoscerate dehydrogenase. Many studies in oncology have been performed showing that 2D electrophoresis can detect differences between normal and cancer cell proteomes, although the extent of these changes appears quite variable. For instance, in breast cancer, only few differences were observed in some studies (10), whereas others found numerous changes that could be used to distinguish normal, benign, and malignant tumours by hierarchical clustering (1, 5). The reasons for such discrepancies are not clear, but can be linked to differences in sample preparation as well as protein staining and quantification rather than to the performance of electrophoresis. 2D gel analysis is still the most established method in protein profiling of complex protein mixtures, although it does have significant drawbacks. It requires relatively large amounts of starting material and can only detect the most abundant proteins, which might be less important than the lower abundant proteins for cancer biomarkers.

The study on proteomic profile of ovine pulmonary adenocarcinoma is the basis for further investigations: using narrower pH range to isoelectric focusing that will allow for better resolution of some proteins and proteomic analysis of blood plasma from JSRV infected sheep. Proposed approach has the potential to identify proteins; specifically those expressed in OPA affected sheep, which could be useful in early diagnosis of the disease.

References


