EuPA Open Proteomics 10 (2016) 42–49

Contents lists available at ScienceDirect

EuPA Open Proteomics

journal homepage: www.elsevier.com/locate/euprot

Comparative proteomic analysis of malignant pleural mesothelioma: Focusing on the biphasic subtype

Laura Giusti[a], Federica Ciregia[a], Alessandra Bonotti[b], Ylenia Da Valle[a], Elena Donadio[a], Claudia Boldrini[a], Rudy Foddis[c], Gino Giannaccini[a], Maria R. Mazzoni[a], Pier Aldo Canessa[d], Alfonso Cristaudo[c], Antonio Lucacchini[a,*]

[a] Department of Pharmacy, University of Pisa, Pisa, Italy
[b] Preventive and Occupational Medicine, University Hospital of Pisa, Pisa, Italy
[c] Department of Translational Research and of New Technologies in Medicine and Surgery, University of Pisa, Pisa, Italy
[d] Dipartimento Ospedaliero Medico 2 dell' ASL5 Spezzino, La Spezia, Italy

ARTICLE INFO

Article history:
Received 18 November 2015
Received in revised form 11 January 2016
Accepted 13 January 2016
Available online 16 January 2016

Keywords:
Mesothelioma
Biphasic
Epithelioid
Proteomics
Serum amyloid
Nrf2

ABSTRACT

Malignant pleural mesothelioma (MPM) is a rare cancer originated from pleural mesothelial cells. MPM has been associated with long-term exposure to asbestos. In this work we performed a comparative proteomic analysis of biphasic pleural mesothelioma (B-PM).

Tissue biopsies were obtained from 61 patients who were subjected to a diagnostic thoracoscopy. 2D/MS based approach was used for proteomic analysis. The 22 proteins found differentially expressed in B-PM, with respect to benign, were analyzed by Ingenuity Pathways Analysis and compared with those obtained for epitheliod pleural mesothelioma (E-PM). A different activation of transcription factors, proteins and cytokines were observed between two subtypes.

1. Introduction

Malignant pleural mesothelioma (MPM) is an asbestos-induced, aggressive tumour, showing resistance to chemo- and radio-therapy and very poor outcome [1,2]. From a cytomorphologic point of view MPM can be distinguished in three different categories including epithelioid, biphasic and sarcomatoid. Biphasic tumours are characterized by the concomitant presence of epithelioid and sarcomatoid cells in close proximity or, more frequently, within distinctly separate areas of a tumour [3]. The response of biphasic tumour to treatment depends on the ratio of these two cellular subtypes. A tumour with the prevalence of sarcomatoid cells is associated with worse prognosis. Although a lot of efforts have been underway, aiming to identify the potential targets for novel therapies, no progress has been made in prolonging the median survival of 1 year from the time of diagnosis [4–6]. MPM is highly resistant to therapy, therefore surgery associated with treatments as radiotherapy and chemotherapy are preferred [7]. So, it is urgent to advance in our knowledge about the disease pathogenesis and to develop more effective therapies for different subtype of mesothelioma. Our group has recently performed a comparative proteome analysis between epithelioid mesothelioma (E-PM) and hyperplasia tissue biopsies. We showed that E-PM samples evidenced an altered expression of nuclear lamin and filament related proteins, in addition to confirming the validity of calretinin as a potential biomarker in the differential diagnosis of MPM [8]. In the present study we extended the comparative proteomic analysis to the biphasic mesothelioma (B-PM) searching for proteins that may play a role in the transition from epithelioid to the most aggressive biphasic phenotype.

2. Materials and methods

2.1. Materials

IPG strips pH 3–10 NL and dry strip cover fluid were purchased from GE Health Care Europe (Uppsala, Sweden). The ECL detection system was purchased from PerkinElmer (MA, USA). Anti-S100A11 (calgizzarin), anti-serum amyloid A1(SAA1) and anti-chloride intracellular channel protein 3 (CLIC3) specific primary antibodies

* Corresponding author at: Department of Pharmacy, University of Pisa, Via Bonanno, 6, 56126 Pisa.
E-mail address: antonio.lucacchini@farm.unipi.it (A. Lucacchini).

http://dx.doi.org/10.1016/j.euprot.2016.01.006

were from Abcam, (Cambridge, UK). Anti-γ enolase (ENO2), was from Cell Signaling Technology (MA, USA). Goat anti-rabbit IgG-HRP conjugate secondary antibody was from stress gene. All other reagents were acquired from standard commercial sources and were of the highest grade available.

2.2. Patients

Sixty-one patients were enrolled at the time of diagnosis before beginning any therapeutic treatment. Tissue biopsies were obtained from patients who were subjected to a diagnostic thoracoscopy. After histological examination, 23 samples were classified as E-PM, 10 samples as B-PM, 14 samples as benign (seven pleural inflammation and seven hyperplasia), and 14 samples as lung carcinoma (nine adenocarcinoma and five squamous cells carcinoma). Benign samples were used as negative control, while lung carcinoma samples were used in the validation step as positive control. Each sample was coded directly at the moment of collection, in order to avoid any personal identification. Table 1 shows the clinical characteristics of patients.

2.3. Ethics statements

The study methodologies were conformed to the standards set by the Declaration of Helsinki and were approved by the Local Ethics Committee and signed consent forms were obtained from all patients.

2.4. Protein preparation from biopsies

Proteins were precipitated from the phenol-ethanol supernatant obtained after treatment with TRIzol® reagent (Life technologies, UK), the resulting pellets were resuspended in rehydration solution (7 M Urea, 2 M thiourea, 4% CHAPS, 60 mM DTT, 0.002% bromophenol blue) and incubated for 30 min at room temperature. After incubation, the samples were centrifuged for 10 min at 14,000 × *g* to remove undissolved material. Protein concentrations were measured with a RC-DC protein assay from Bio-Rad, using bovine serum albumin as standard. All samples were stored at −80 °C until analysis.

2.5. 2D gel electrophoresis

According to histological analysis B-PM, E-PM and benign samples were used to constitute respectively three different pools for 2D analysis. IEF was carried out by using 18 cm Immobiline Dry-Strips (GE Healthcare) with a nonlinear, pH 3–10, gradient. Two-hundred micrograms of proteins were filled to 400 μL with rehydration buffer supplemented with 1.2% v/v IPG Buffer, pH 3–10 NL (GE Healthcare). IEF was performed at 16 °C on an Ettan IPGphor II apparatus (GE Healthcare) according to the previously described schedule [9]. After IEF, the strips were equilibrated as described, and SDS-PAGE was performed using the PROTEAN-II Multi Cell system (Bio-Rad) [10]. The gels were performed in triplicate.

2.6. Staining and image analysis

The gels were stained with Ruthenium II tris (bathophenanthroline disulfonate) tetrasodium salt (SunaTech Inc.) essentially as described by Aude-Garcia et al [11] with minor modifications [8,10].The acquisition on fluorescence of all gel images was performed by "ImageQuant LAS4010" (GE Healthcare). The images were analyzed with the SameSpots (version 4.1.3978., TotalLab, Ltd., UK) software as previously described [12]. The protein spots with a ≥2 fold of spot quantity change, $p < 0.05$ and q-value < 0.05 were selected and identified.

2.7. MS analysis and protein identification

Spots of interest were cut out from gel reference and the nano-LC-ESI-MS/MS analysis by LTQ-Orbitrap Velos was performed as previously described [10,12] Peak lists were generated from raw orbitrap data using the embedded software from the instrument vendor (extract_MSN.exe). The monoisotopic masses of the selected precursor ions were corrected using an inhouse written Perl script [13]. The peak list files were searched against the SwissProt/trEMBL database (Release 2013 03 of 06 March 2013) using Mascot (Matrix Sciences, London, UK). Human taxonomy (98 529 sequences) was specified for database searching. The parent ion tolerance was set at 10 ppm. Oxidation of methionine was specified in Mascot as a variable modification. Trypsin was selected as the enzyme, with one potential missed cleavage, and the normal cleavage mode was used. The mascot search was validated using Scaffold 4.4.5 (Proteome Software, Portland, OR). Only proteins matching with two different peptides with a minimum probability score of 95% were considered to be identified. The FDR at protein and peptide level was 0.0% and 0.2% respectively (Prophet). The reference limit to $p < 0.05$ for the probabilistic scores of MS/MS assignment was 45. When multiple proteins were identified in a single spot, the proteins with the highest number of peptides were considered as those corresponding to the spot.

2.8. Western blot analysis

For 1D western blot (WB), all samples were processed to validate different protein expressions found with 2D analysis. Aliquots (25 μg of proteins) of each sample (23 E-PM, 10 B-PM, 14 benign, and 14 lung carcinoma) were solubilized with a SDS sample buffer (Laemmli solution), and proteins were separated by 12% SDS-PAGE under reducing conditions and WB was carried out as previously described [14]. Before blocking the nitrocellulose, membranes were reversibly stained with 1 mM RuBP as previously described [10]. Specific primary antibodies were diluted in blocking buffer as follows: 1:2000 for anti-S100A11, anti-CLIC3, and anti-SSA1 and 1:500 anti-γ-enolase. The immunocomplexes were detected using a peroxidase labeled secondary antibody (goat anti-rabbit, 1:10000 dilution). Immunoblots were developed using the ECL detection system. The chemiluminescent images were acquired by LAS4010 (GE Healthcare). The experiments were performed in duplicate. Normalization was performed on total proteins loaded for each sample.

2.9. Statistical analysis

Statistical analysis of the three classes (B-PM, E-PM, and benign) of gels was performed by Same Spot (TotalLab, Ltd., UK). The software included the following statistical analysis calculations: Anova p-value, and false discovery rate (q-values). The OD of the proteins was expressed as a percentage of the volume (mean ± SEM). For the comparison of protein expression levels between different subtype of MPM and with respect to control samples (benign and carcinoma samples), the antigen-specific bands were quantified using the Image Quant-L (GE Healthcare). The significance of the differences (p-value ≤ 0.05) was calculated by the Mann–Whitney test.

2.10. Signaling pathway analysis

Proteins differentially expressed, were functionally analyzed through the use of QIAGEN's Ingenuity Pathway Analysis (IPA,