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The secretome signature of malignant mesothelioma cell lines

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ABSTRACT

The secretome is the complex set of molecules secreted by cells; these molecules play a key role in cell signaling, communication and migration. Secretomics has been already used to discover new potential diagnostic biomarkers and therapeutic agents and to elucidate key autocrine pathways. Malignant mesothelioma (MMe), an extremely aggressive tumor, is characterized by a long latency period (20–30 years), a poor prognosis, and limited effective therapies. MMe has a highly secretory cell type, and the factors released by cells may act in an autocrine or paracrine fashion on tumor and stroma, where they may modulate the extracellular environment. The aim of this work is to characterize the secretome of two MMe cell lines, MM98 and REN, in comparison with a mesothelial cell line Met5A, in order to evaluate differences and similarities of these two different MMe cancer model systems, and to identify potential biomarkers. We performed quantitative shotgun proteomics using SWATH-MS technology and we identified a total of 421 proteins, 112 expressed in the secretome of REN cells, 208 expressed in the secretome of MM98 cells and 189 secreted by mesothelial cells; 25 proteins are shared by the two mesothelioma cell lines.

Biological significance: This study characterizes the secretome signature of the REN and MM98 cell lines, confirming the availability of a cell-culture based model in order to describe the cell-specific properties, and to provide a list of putative cancer biomarkers.

This work constitutes the first qualitative and quantitative proteomic approach performed on MMe secretome. Moreover, since the data were acquired in SWATH-MS acquisition mode, they can be successively re-mined without performing a new analysis of the sample, which is extremely useful for retrospective analyses.

The overall aim was to identify novel tumor-derived protein biomarkers with the potential to be applied for early diagnosis, prognosis, therapy prediction and/or disease monitoring of MMe.

1. Introduction

Malignant mesothelioma (MMe) is an extremely aggressive tumor characterized by a long latency period (20–30 years), a poor prognosis, and limited effective therapies. Exposure to asbestos is the major cause, with occupational exposure being documented in 70–80% of MMe patients [1,2].

MMe is sub-typed into three forms according to the histological morphology: epithelial, sarcomatoid, and biphasic. The sarcomatous type is the less frequent, but the most aggressive and rapidly fatal [3]. Despite the last promising results made on the development of new therapies [4–6], long-term survival with currently available treatment is rare. Therefore, new therapies for MMe are urgently needed.

The diagnosis of MMe is challenging, due to similarities in the clinical presentation and histological features of MMe to primary lung carcinoma, pleural metastases, reactive pleural diseases and rare pleural malignancies [7].

The current gold standard of MMe diagnosis is a combination of two positive and two negative immunohistochemical markers in the epithelioid and biphasic type, but sarcomatous type does not have specific markers, making diagnosis more difficult. Circulating proteomic and microRNA signatures, tumor cell gene-ratio test, transcriptomics, lncRNA, glycopeptides and pleural fluid FISH assay may be important future markers [7].

Early diagnosis of MMe would increase the overall survival, but preclinical biomarkers are not yet available [1].

The secretome consists of proteins that are secreted and shed from the cell surface and intracellular proteins released into the medium due to cell lysis, necrosis and apoptosis [8].

MMe has a highly secretory cell type, and the factors released by cells may act in an autocrine or paracrine fashion on tumor and stroma, where they may modulate the extracellular environment.

The secretome represents a good opportunity for discovering proteins involved in cancer [9], and in addition, its limited complexity, compared with serum and plasma, enhances the possibility to identify less

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abundant proteins [10]. The interaction of sarcomatous MMe with the extracellular matrix has already been investigated, but only few proteins were identified [11].

Few works analyzed the secretome of MMe cell lines. The analysis of the effusion proteome of MMe patients identified novel potential diagnostic biomarkers like galectin-1 [12], while the analysis of MMe by laser microdissection and proteomics highlighted the role of cathepsin D as potential biomarker against the most popular lung cancers [13].

The use of SOMAmer proteomic technology provided potential biomarkers for the diagnosis of MMe [14] and the study of N-glycoproteins from surfaceome of MMe explored the possibility to suggest new biomarker candidates [15,16].

The protein set secreted in the conditioned media (CM) of a serum-free cellular culture represents the signature of a specific cell line and it is peculiar of the organ/tissue of origin.

Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) is the best analytical method for the identification and quantification of proteins in biological samples [17–20]. Sequential window acquisition of all theoretical fragment-ion spectra mass spectrometry (SWATH-MS) is a data independent analysis (DIA) workflow that uses a first quadrupole isolation window to step across a mass range, collecting high resolution full scan composite MS/MS spectra at each step, and generating an ion map of the fragments from all the detectable precursor masses [21,22].

The aim of the present study is to characterize the secretome of two human MMe cell lines, namely REN and MM98, by shotgun proteomics and SWATH-MS, in order to evaluate the differences and similarities of the two MMe model systems. We have also compared the secreted proteins of MMe model systems with normal mesothelial cell line Met5A.

2. Materials and methods

2.1. Cell culture

Two MMe cell lines were used: REN cells, a p53 mutant epithelial subtype [23] and MM98 cells, established from pleural effusion of a sarcomatous mesothelioma [24] and one cell line of human mesothelial cells (Met5A from ATCC).

These cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS, Euroclone, Pero, Italy) and 1% antibiotic mixture (Sigma-Aldrich), and maintained at 37 °C in humidified atmosphere with 5% CO_2.

2.2. Isolation of secreted proteins

When cells reached the 60–70% confluence, the cell monolayer was gently washed twice with PBS 1× and once with F10-Ham without serum. Then, the cells were incubated in F10-Ham without serum at 37 °C. After 24 h, the conditioned media containing the secreted proteins were collected and filtered using a 0.45 μM filter to remove detached cells and large debris. The supernatants were collected and then subjected to TCA (12%) precipitation at 4 °C. After 4 h, the solutions were precipitated and the pellets were washed twice with THF [18]. Finally, the pellets were redissolved in 100 mM ammonium bicarbonate solution.

2.3. In-solution digestion

The proteins were reduced and alkylated, then digested with trypsin overnight (Sigma-Aldrich) [20].

2.4. SWATH-MS analysis

LC–MS/MS analyses were performed using a micro-LC Eksigent Technologies (Dublin, USA) system with a stationary phase of a Halo Fused C18 column (0.5 × 100 mm, 2.7 μm; Eksigent Technologies, Dublin, USA). The injection volume was 5.0 μL and the oven temperature was set at 40 °C. The mobile phase was a mixture of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B), eluting at a flow-rate of 15.0 mL min^{-1} at an increasing concentration of solvent B from 2% to 40% in 30 min. The LC system was interfaced with a 5600 + TripleTOF system (AB Sciex, Concord, Canada) equipped with a DuoSpray Ion Source and CDS (Calibrant Delivery System). Samples used to generate the SWATH-MS spectral library were subjected to the traditional data-dependent acquisition (DDA): the mass spectrometer analysis was performed using a mass range of 100–1500 Da (TOF scan with an accumulation time of 0.25 s), followed by a MS/MS product ion scan from 200 to 1250 Da (accumulation time of 5.0 ms) with the abundance threshold set at 30 cps (35 candidate ions can be monitored during every cycle). The samples were then subjected to cyclic data independent analysis (DIA) of the mass spectra, using a 25-Da window [29–31]: the mass spectrometer was operated such that a 50-ms survey scan (TOF-MS) was performed and subsequent MS/MS experiments were performed on all precursors. These MS/MS experiments were performed in a cyclic manner using an accumulation time of 40 ms per 25-Da swath (36 swaths in total) for a total cycle time of 1.5408 s. The ions were fragmented for each MS/MS experiment in the collision cell using the rolling collision energy. The MS data were acquired with Analyst TF 1.7 (AB SCIEX, Concord, Canada). Three replicates for each sample were subjected to the DIA analysis.

2.5. Protein database search

The mass spectrometry files were searched using Protein Pilot (AB SCIEX, Concord, Canada), Mascot (Matrix Science Inc., Boston, USA) and the Trans-Proteomic Pipeline (TPP). Samples were input in the Protein Pilot software v. 4.2 (AB SCIEX, Concord, Canada), which employs the Paragon algorithm, with the following parameters: cysteine alkylation, digestion by trypsin, no special factors and False Discovery Rate at 1%. The search was conducted using the UniProt Swiss-Prot database containing human proteins (version 2015.06.09, containing 20,207 sequence entries). The Mascot search was performed on Mascot v. 2.4, the digestion enzyme selected was trypsin, with 3 missed cleavages and a search tolerance of 0.4 Da was specified for the peptide mass tolerance, and 0.4 Da for the MS/MS tolerance. The charges of the peptides to search for were set to 2 +, 3 + and 4 +, and the search was set on monoisotopic mass. The instrument was set to ESI-QUAD-TOF and the following modifications were specified for the search: carbamidomethyl cysteins as fixed modification and oxidized methionine as variable modification. The TPP search and validation of the identified peptides and proteins were carried out according to the manual of the TPP software, and validated with PeptideProphet and ProteinProphet. Dual filtering criteria for protein identification were employed by combining FDR test from target-decoy database search with a cutoff *p*-value of 0.05, and protein/peptide confidence above 95% probability, with a minimum of two unique peptides per protein.

2.6. Library generation for the SWATH analysis

The label-free quantification was performed with Skyline (MacCoss Lab Software, University of Washington) by importing the SWATH-MS runs. The library of the identified proteins used for the processing of SWATH data was generated by combining the results of the database search performed with Protein Pilot, Mascot and TPP. The files .group from Protein Pilot, .dat from Mascot and .pep.xml from TPP were loaded into Skyline, generating a single library. The quantification was performed by integrating the extracted ion chromatogram of all the unique ions for a given peptide, wherefore unique ions are intended for the ions that belong to only one of the possible isobaric species.

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