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Proteomic analysis of formalin-fixed, paraffin-embedded lung neuroendocrine tumor samples from hospital archives

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ABSTRACT

Hospital tissue repositories host an invaluable supply of diseased samples with matched retrospective clinical information. In this work, a recently optimized method for extracting full-length proteins from formalin-fixed, paraffin-embedded (FFPE) tissues was evaluated on lung neuroendocrine tumor (LNET) samples collected from hospital repositories. LNETs comprise a heterogeneous spectrum of diseases, for which subtype-specific diagnostic markers are lacking. Six archival samples diagnosed as typical carcinoid (TC) or small cell lung carcinoma (SCLC) were subjected to a full-length protein extraction followed by a GeLC–MS/MS analysis, enabling the identification of over 300 distinct proteins per tumor subtype. All identified proteins were categorized through DAVID software, revealing a differential distribution of functional classes, such as those involved in RNA processing, response to oxidative stress and ion homeostasis. Moreover, using spectral counting for protein abundance estimation and beta-binomial test as statistical filter, a list of 28 differentially expressed proteins was generated and submitted to pathway analysis by means of Ingenuity Pathway Analysis software. Differential expression of chromogranin-A (more expressed in TCs) and stathmin (more expressed in SCLCs) was consistently confirmed by immunohistochemistry. Therefore, FFPE hospital archival samples can be successfully subjected to proteomic investigations aimed to biomarker discovery following a GeLC–MS/MS label-free approach.

1. Introduction

Formalin-fixed, paraffin-embedded (FFPE) tissues stored in hospital repositories worldwide represent a powerful resource for biomarker discovery. In fact, a large availability of archival specimens is associated with valuable clinical history information (including therapy and follow-up data), thus allowing large retrospective investigations. Additionally, FFPE tissues facilitate the long-distance exchange of samples, being stable and biohazard-free, and pose very limited ethical issues

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compared to fresh tissues [1,2]. However, a significant hindrance to their use for performing proteomic studies is the extensive crosslinking introduced by formaldehyde on proteins. In the last few years, several shotgun proteomics studies have been published exploiting high-temperature extraction followed by direct trypsin digestion of FFPE extracts [3–8]. Recently, we reported an optimized extraction method enabling recovery of full-length proteins from FFPE tissues [9]. The successful application of proteome analysis techniques, including gel-based approaches, such as GeLC–MS/MS [9], 2-D PAGE-MS [10], and 2-D DIGE (Tanca et al., in press), was demonstrated using animal model tissues. However, the performance of this methodological approach still needs to be evaluated on human samples from hospital repositories, and quality and robustness of the results have not been fully assessed to date.

The ability to investigate FFPE samples would open new research possibilities in diseases for which samples are difficult to be retrieved, such as those having high heterogeneity, low subtype incidence, or which are routinely treated with surgical therapies without recovery of bioptic samples. Lung neuroendocrine tumors (LNETs) are a good candidate for evaluating the performance of a gel-based proteomic approach on FFPE tissues. LNETs account for about 20% of all pulmonary cancers, but, being so diversified, their subtype-specific incidence is low. These tumors encompass a spectrum of neoplastic lesions arising from neuroendocrine cells of the pulmonary epithelium, but are conventionally classified into four subgroups: typical carcinoid tumor (TC), atypical carcinoid tumor (AC), large-cell neuroendocrine carcinoma (LCNEC), and small-cell lung carcinoma (SCLC) [11,12]. TCs are considered as low-grade malignancies with favorable prognosis which may benefit from complete surgical resection, whereas SCLCs show a highly aggressive behavior and are usually treated with chemotherapy without surgery. Therefore, an accurate differential diagnosis among these variants is mandatory for a correct choice of therapy. Yet, mistaken diagnosis of SCLCs has been reported as one of the most important "pitfalls" in the management of lung cancers, often dependent on the small size of the samples obtained from bronchoscopy or CT-assisted biopsy [13]. To date, a large number of markers, including Ki-67, chromogranin A (CgA), neuron-specific enolase, serotonin, synaptophysin, and adrenocorticotrophic hormone, are currently used by pathologists in combination with morphological analysis. However, differential diagnosis remains cumbersome, since none of these markers has a satisfactory degree of sensitivity and specificity. For instance, increased plasma CgA level has been proposed as a diagnostic tool for lung carcinoids, but it is also observed in up to 60% of SCLC cases [11].

Some gene expression studies on LNET samples were carried out in the past few years [14–20]; on the other hand, proteomic characterization of LNETs lags considerably behind: only a limited comparative 2-D PAGE survey was conducted in 2005 by Cho et al. [21]. A thorough differential proteomic investigation, particularly comparing TC and SCLC, might lead to discovery of novel biomarkers for the improvement of diagnostic efficiency. As far as translational research is concerned, the largest hindrance to proteomic profiling of LNETs is represented by their low subtype-specific incidence, especially for carcinoids [11]. Indeed, the collection of fresh/frozen tissue samples in adequate amount and quality for a differential proteomics study would require a concerted action of surgeons, pathologists, and laboratory personnel during a long-lived research project.

Based on the experience accumulated in our laboratory with animal model samples, a GeLC–MS/MS differential proteomic analysis approach was applied to TC and SCLC FFPE archival biopsies, and the performance of the spectral counting approach for identification of differentially abundant proteins was assessed.

2. Materials and methods

2.1. Samples

Formalin-fixed, paraffin-embedded tissue blocks from 6 LNETs surgical specimens, comprising 3 TCs and 3 SCLCs, were retrieved from the tissue repositories of the Departments of Pathology at the University Hospitals of Sassari and Verona, Italy. Tissue blocks had been stored for 4 to 60 months before analysis. Hematoxylin and eosin stains were critically reviewed and the tumors were classified according to the WHO 2004 classification of neuroendocrine neoplasm of the lung [12]. Representative FFPE tissue blocks were then selected to assure the presence of at least 80% of neoplastic tissue, and consecutive sections were obtained for proteomic assays and immunohistochemical analyses.

2.2. Protein extraction and quantification

Protein extraction from FFPE tissues was performed as described previously [9]. Briefly, microtome sections (10 μm thick, 80 mm^2 wide) were cut from FFPE tissue blocks and deparaffinized by incubation in xylene, rehydrated with a graded series of ethanol, immersed at a 20% w/v ratio in SDS extraction buffer, and subjected to high-temperature extraction. Protein extracts were clarified for 15 min at 12,000×*g* at 4 °C, quantified by the EZQ Protein Quantification Kit (Molecular Probes, Eugene, OR), and stored at –80 °C until needed.

2.3. GeLC–MS/MS analysis

About 25 μg of proteins extracted from FFPE tissues were subjected to SDS-PAGE according to Laemmli [22]. Gels were stained with Coomassie Brilliant blue G-250 [23], and digitalized with an ImageScanner III (GE Healthcare). Visible protein bands were excised or, alternatively, the whole lane was fractionated into gel slices. Gel slices were destained, reduced, carbamidomethylated, and trypsin digested as described previously [9]. Briefly, samples were reduced in 50 mM NH_4HCO_3 buffer with 10 mM DTT at 56 °C, and then carbamidomethylated in 50 mM NH_4HCO_3 buffer with 55 mM iodoacetamide at room temperature in the dark. Tryptic digestion was performed at 37 °C overnight using an average amount of 80 ng of trypsin per gel slice.

LC–MS/MS analyses of tryptic digests were performed on a Q-TOF hybrid mass spectrometer equipped with a nano lock Z-spray source, and coupled on-line with a capillary