JOURNAL OF PROTEOMICS 112 (2015) 250–261

Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jprot

Comparison of two FFPE preparation methods using label-free shotgun proteomics: Application to tissues of diverticulitis patients

Florence Quesada-Calvo[a], *Virginie Bertrand*[b], *Rémi Longuespée*[c], *Agnès Delga*[d], *Gabriel Mazzucchelli*[c], *Nicolas Smargiasso*[c], *Dominique Baiwir*[e], *Philippe Delvenne*[f], *Michel Malaise*[g], *Marie-Claire De Pauw-Gillet*[b], *Edwin De Pauw*[c], *Edouard Louis*[a], *Marie-Alice Meuwis*[a,*]

[a]*Gastroenterology Department, GIGA-R, Liège University Hospital CHU, ULg, Liège, Belgium*
[b]*Department of Preclinical and Biomedical Sciences, Mammalian Cell Culture Laboratory, GIGA-R, ULg, Liège, Belgium*
[c]*Chemistry Department, Laboratory of Mass Spectrometry, GIGA-R, CART, ULg, Liège, Belgium*
[d]*Biobanque universitaire, Liège University Hospital CHU, Liège, Belgium*
[e]*GIGA Proteomic Facility, ULg, Liège, Belgium*
[f]*Department of Preclinical and Biomedical Sciences, Laboratory of Cytology and Histology, GIGA-R, ULg, Liège, Belgium*
[g]*Department of Clinical Sciences, Department of Rheumatology, Liège University Hospital CHU, Liège, Belgium*

ARTICLE INFO

Article history:
Received 22 April 2014
Accepted 28 August 2014
Available online 10 September 2014

Keywords:
FFPE
Antigen retrieval
Filter aided sample preparation (FASP)
Shotgun proteomics
Label-free
Data independent

ABSTRACT

Formalin-fixed paraffin-embedded (FFPE) specimens of patients are useful sources of materials for clinical research and have recently gained interest for use in the discovery of clinical proteomic biomarkers. However, the critical step in this field is the ability to obtain an efficient and repeatable extraction using the limited quantities of material available for research in hospital biobanks. This work describes the evaluation of the peptide/protein extraction using FFPE sections treated by the following two methods before shotgun proteomic analysis: a commercial solution (FFPE–FASP) (filter aided sample preparation) and an antigen retrieval-derived protocol (On Slice AR). Their efficiencies and repeatabilities are compared using data-independent differential quantitative label-free analysis. FFPE–FASP was shown to be globally better both qualitatively and quantitatively than On Slice AR. FFPE–FASP was tested on several samples, and differential analysis was used to compare the tissues of diverticulitis patients (healthy and inflammatory tissues). In this differential proteomic analysis using retrospective clinical FFPE material, FFPE–FASP was reproducible and provided a high number of confident protein identifications, highlighting potential protein biomarkers.

Biological Significance
In clinical proteomics, FFPE is an important resource for retrospective analysis and for the discovery of biomarkers. The challenge for FFPE shotgun proteomic analysis is preparation by an efficient and reproducible protocol, which includes protein extraction and digestion. In this study, we analyzed two different methods and evaluated their repeatabilities and efficiencies. We illustrated the reproducibility of the most efficient method, FFPE–FASP, by a

* *Corresponding author.*
E-mail address: marie-alice.meuwis@ulg.ac.be (M.-A. Meuwis).

http://dx.doi.org/10.1016/j.jprot.2014.08.013

pilot study on diverticulitis tissue and on FFPE samples amount accessible in hospital biobanks. These data showed that FFPE is suitable for use in clinical proteomics, especially when the FFPE–FASP method is combined with label-free shotgun proteomics as described in the workflow presented in this work.

1. Introduction

Formalin-fixed paraffin-embedded (FFPE) tissues stored in hospital biobanks represent a valuable resource for retrospective analysis. Hence, larger populations can be studied, increasing the possibility of identifying significant and specific potential biomarkers. FFPE tissues are advantageous in many ways when compared to fresh frozen specimens; they can be stored for very long periods of time and are easier to obtain as they are collected for systematic routine diagnosis. However, the formalin fixation of tissue involves the creation of inter- and intra-molecular crosslinks [1,2]. Therefore, one of the challenges for proteomic studies consists of extracting the highest number of different proteins present in the sample and obtaining confident protein identifications. Many protocols exist for "unlocking" FFPE crosslinks induced by the formalin fixation of tissue. These involve protein solubilization and digestion prior to analysis using various techniques including proteomics [3,4,1,5–8]. These techniques were recently reviewed [1,9,10]. Among these methods, the citric acid antigen retrieval (CAAR) method, which was initially developed for imaging by mass spectrometry (IMS), was adapted using laser capture microdissection (LCM) before shotgun proteomics [4,11]. The advantage of the CAAR method is that it is performed on slices mounted on glass slides allowing conservation of the 2D spatial resolution for IMS. Applying an AR strategy using a tissue section on a slide for FFPE preparation before shotgun proteomics is rarely described in the literature [11].

Some papers commented on the stability and quality of FFPE material over time, the variability of the fixation protocols, the capacity to identify the same range of proteins as with fresh frozen samples and the possibility for studying posttranslational modifications such as glycosylation and phosphorylation [7,12–15]. However, the variability of these "unlocking" protocols on the quality of results, mostly regarding the quantitative reproducibility or repeatability, was seldom commented on.

In the context of the discovery of disease biomarkers, various studies addressed FFPE specimens, and some used differential analysis by label-free shotgun proteomics [16,17,14]. One of the instrument systems enabling a differential label-free proteomics study of complex samples with high sensitivity, high specificity and a linearity over 4 orders of magnitude in terms of the dynamic range of the protein concentration is the 2D-*nano* UPLC (ultra-performance liquid chromatography) Q-Tof Synapt HDMS™ G2 system (Waters Corporation, Milford, USA). This data-independent MS^E offers some gains over traditional data-dependent analysis, including good measurement reproducibility, identification of low-abundance peptides, faster throughput due to the simultaneous fragmentation of multiple peptides and direct relative quantification [18].

Translational research by shotgun proteomics for the discovery of biomarkers might involve the use of pooled sample extracts [19–21]. Hence, working on pools somehow results in a smoothing out of characteristics with high biological variability linked to demographic differences of the patients. The biological variability can also be driven by the clinical staging of diseased samples, the selection of the specimens, the surfaces of cells and tissues treated (with or without micro- or macrodissection), the sample treatments before the FFPE process and differences in the storage of the samples. All of these parameters have to be carefully considered and controlled when composing balanced pools to avoid bias in the selection of samples. Tissue biobanks involve the collection and storage of residual material used first for the diagnosis of patients. Hence, some pathological stages or grades are, by nature, rather tiny specimens (such as some colorectal adenoma polyps). Therefore, limited quantities of materials are available for translational research.

These limitations and precautions drove us to perform the preliminary study presented in this paper comparing the respective performances (efficiencies and repeatabilities) of two FFPE preparation protocols using differential label-free quantitative analysis to select the best option for application to a clinical study. Hence, a few slices of a FFPE specimen of the same colorectal cancer patient were treated by the following two methods in triplicate: the commercial FFPE–FASP (filter-aided sample preparation) kit and an on slice antigen retrieval-derived protocol ("On Slice AR"). Finally, to evaluate its reproducibility, the FFPE–FASP method was applied on FFPE tissue samples of diverticulitis patients for comparison of the proteins obtained within tissue areas with inflammation to those obtained from within matched healthy zones.

2. Materials and methods

Fig. 1A shows the workflow of the experimental strategy steps followed in our work for the two FFPE preparation protocols that were tested and compared.

2.1. Tissue samples

Human tissue samples were obtained from the Biobank of the Liège University Hospital, and the research protocol was approved by the Ethics Committee of our University Hospital. The tissue specimens were processed using standard procedures for formalin fixation (length of 24 h) and embedded in paraffin as done for routine clinical analysis [22]. Six-micrometer-thick sections were placed on glass slides and stained with hematoxylin and eosin for the identification of histologically distinct tissue regions. For the FFPE–FASP and On Slice AR tests, the selected specimen tissue block originated from a patient with colorectal cancer (pT3N0Mx). The FFPE tissue serial sections performed using this specimen were grouped in an alternating manner to homogenize the biological material available for the six analyses (three replicates per FFPE preparation method) as much as possible.