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A laser microdissection-based workflow for FFPE tissue microproteomics: Important considerations for small sample processing

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ABSTRACT

Proteomic methods are today widely applied to formalin-fixed paraffin-embedded (FFPE) tissue samples for several applications in research, especially in molecular pathology. To date, there is an unmet need for the analysis of small tissue samples, such as for early cancerous lesions. Indeed, no method has yet been proposed for the reproducible processing of small FFPE tissue samples to allow biomarker discovery. In this work, we tested several procedures to process laser microdissected tissue pieces bearing less than 3000 cells. Combined with appropriate settings for liquid chromatography mass spectrometry–mass spectrometry (LC–MS/MS) analysis, a citric acid antigen retrieval (CAAR)-based procedure was established, allowing to identify more than 1400 proteins from a single microdissected breast cancer tissue biopsy. This work demonstrates important considerations concerning the handling and processing of laser microdissected tissue samples of extremely limited size, in the process opening new perspectives in molecular pathology. A proof of the proposed method for biomarker discovery, with respect to these specific handling considerations, is illustrated using the differential proteomic analysis of invasive breast carcinoma of no special type and invasive lobular triple-negative breast cancer tissues. This work will be of utmost importance for early biomarker discovery or in support of matrix-assisted laser desorption/ionization (MALDI) imaging for microproteomics from small regions of interest.

1. Introduction

Since tissue material contains all information on proteomic and genetic changes in physiological and pathological conditions, it represents the best possible sample material for molecular research in life science. Formalin fixation preserves tissue integrity and thereby greatly improves tissue processing and storage quality, and is therefore adopted in all pathology laboratories. Several procedures were already designed in order to use formalin-fixed paraffin-embedded (FFPE) tissues in proteomics [1,2]. Laser microdissection (LMD) is a method to collect small tissue regions that can subsequently be analyzed by proteomic techniques

Abbreviations: FFPE, formalin-fixed paraffin-embedded; AR, antigen retrieval; SDS, sodium dodecyl sulfate; MALDI MSI, matrix-assisted laser desorption/ionization mass spectrometry imaging; CA, citric acid; CAAR, citric acid antigen retrieval; TNBC, triple negative breast cancer; NST, no special type; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; RG, RapiGest; RG, MassPREP Digestion Standards Mixtures; H&E, hematoxylin and eosin; DTT, dithiothreitol; CAN, acetonitrile; TFA, trifluoroacetic acid; LC–MS/MS, liquid chromatography mass spectrometry–mass spectrometry; LFQ, label free quantification; LIMA1, LIM actin-binding protein 1; IRS, immunoreactive score; PSM, peptide-spectrum matches; fr/fr, fresh/frozen; EC, endocervix; HSIL, high-grade squamous intraepithelial lesion; RPM, revolutions per minute; RCF, relative centrifugal force.

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[1,3,4]. Up to now, these procedures were mostly applied on relatively large tissue areas. Early events of cancers are generally restricted to very small tissue regions. However, there is still an unmet need for biochemical preprocessing methods to analyze FFPE samples of limited size by proteomics.

In the present study, we tested chemical handling procedures for microdissected FFPE cancerous tissue pieces, bearing 2700 ± 245 cells, based on a breast cancer biopsy as study model. We highlight mandatory precautions to avoid sample loss, which is a critical issue when small tissue biopsies are analyzed. It was absolutely essential to take these considerations into account to design a method that fitted the needs for biomarker discovery from very small FFPE tissues. The approach is based on the use of citric acid (CA) as a solution for heat-induced antigen retrieval (AR). Heat-induced AR procedures were developed for immunohistochemistry (IHC) in the early 1990s by Shi et al. [5], based on studies published in the 1940s [6,7]. These studies indicated that cross-linkage between formalin and proteins during fixation could be disrupted by heating at 100 °C and above. Although it is generally considered that the temperature is more important than the antigen retrieval solution itself, pH adjustment can lead to more or less efficient immunohistochemical staining [8]. Despite the fact that detergent solutions such as sodium dodecyl sulfate (SDS) are commonly used for tissue proteomics, it is generally applicable for the lysis of large amounts of tissues [9]. For studies requiring a carefull preservation of the tissue such as for matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI), IHC solutions are more adequate. In 2009, Hoffmann et al. [11] compared two solutions for FFPE ovarian cancer tissue sections processing prior to MALDI imaging analyses i.e. the previously known Tris/EDTA pH9 [10] and the CA pH 6 solution. They proved that citric acid antigen retrieval (CAAR), compared to Tris/EDTA AR (both combined with tryptic digestion) allowed to retrieve more proteolytic peptides [11]. In this study, the authors also showed that the method gave similar results when using 1 or 17 years old FFPE tissue blocks. Even if not clearly proved, it can be speculated that the acidic pH can favor the formation of positively charged residues on basic amino acids and, by charge repulsion, enhance the proteins' unfolding during antigen retrieval without any specificity for a given epitope. In 2013, Longuespée et al. successfully applied this procedure in a shotgun proteomic study to decipher the histological etiology of serous ovarian cancers [12].

The CAAR-based method was checked for its reproducibility and applied to a panel of 7 cancerous tissues from various origins. The final protocol allowed us to obtain from 890 up to 1447 protein identifications depending on the tissue type.

The validity of the biomarker discovery method was tested in an assay comparing triple negative breast cancer (TNBC) samples. Despite molecular stratification efforts [13], immunohistochemical analyses are still the golden standard to determine the clinically relevant subtypes of breast cancer depending on estrogen-, progesterone receptor and HER2 reactivity as well as the proliferation indices, namely luminal A, luminal B, HER2 positive and triple-negative. Triple-negative breast cancer tumors are associated with an adverse prognosis [14] and are not eligible for targeted therapy, unlike luminal or HER2 positive breast cancer [15].

Invasive carcinoma of no special type (NST), previously known as invasive ductal carcinoma (IDC), and invasive lobular carcinoma (ILC) of the breast represent the two most common subtypes of invasive breast cancer (BC) (80% of all invasive BCs). Both subtypes may exhibit a similar histomorphological picture, especially in small core biopsies. In these cases, the diagnosis is supported by immunohistological analyses [16]. Some studies highlighted that the metastatic profile and the prognosis may differ depending on the histological type [17]. It also appears that for the same neoadjuvant chemotherapy, IDC has a better rate of complete pathological response after conservative surgery than ILC [18]. It is well known that a good immune response improves the effectiveness of different cancer treatments [19], but this only seems to have a prognostic value in ductal carcinomas [20]. The histological subtype may then influence the prognosis of breast cancer. This prognostic difference suggests the existence of different molecular mechanisms. In this study, TNBC IDC and ILC were analyzed and compared with the designed workflow, as a proof of concept for biomarker discovery.

Finally, one of the promising applications of handling small amounts of FFPE tissues is illustrated by the analysis of metaplastic and dysplastic cervical tissues. The number of cells in that kind of tissues is very limited and it would make sense to use microproteomics avoiding tissue loss during processing, for this type of application. Biomarker discovery of early events of cancerization is of particular interest in order to find new diagnoses and therapeutic targets when it is still at a curable stage. This procedure may offer new opportunities for early biomarkers research and may be applied to new types of investigations in pathology.

2. Experimental procedures

2.1. Material

Most solvents were purchased from Biosolve (Dieuze, France); CA, formic acid, SDS and NH_4HCO_3 from Fluka/Sigma Aldrich (Diegem, Belgium). RapiGest (RG) and MassPREP Digestion Standards Mixtures (MPDS Mix) were from Waters (Zellik, Belgium), polyethylene naphthalate microdissection membrane slides from Leica (Diegem, Belgium). A clean-up kit for SDS removal was provided by GE Healthcare (Diegem, Belgium). Trypsin Protease MS Grade was purchased from Pierce (Thermo Fischer Scientific, Landsmeer, Netherlands). Axygen 0.6 mL tubes (Fisher Scientific, Aalst, Belgium) were used for tissue collection/processing and ZipTip cartridges were provided by Milipore (Overijse, Belgium). Anti-LIMA1 antibody was provided by Sigma Aldrich (St. Louis, USA). The secondary antibody kit Optiview DAB IHC Detection Kit was provided by Ventana (Tucson, USA).

2.2. Samples

Tissues were provided by the University of Liège with institutional ethical review approval (MSI/MICROPROT1). Cancer tissues originating from cervix, cervix cancer cells metastasis in sentinel lymph node, kidney, lung, liver and breast were analyzed. After surgery, the tissues were fixed overnight in 10% formalin and dehydrated first in methanol and then in isopropanol, 3 baths each.

Fixed dehydrated tissues were then embedded in warm paraffin and stored in FFPE anatomopathological tissue holders. FFPE blocks were selected by our reference pathologist Philippe Delvenne and 4–5 µm tissue sections were cut with a Thermo HM340 microtome and dropped on the surface of a water bath. Individual sections were then deposited either on PEN membrane slides for microdissection or on Superfrost glass slides for further hematoxylin and eosin (H&E) staining. Next, the tissues were heated at 60 °C for 2 h and bathed twice in xylene for 5 min for tissue dewaxing. To ensure xylene removal and tissue rehydration, successive bathes of 100% isopropanol were performed for 2 min. Finally, the tissue sections were dried and stored at 4 °C before laser microdissection.

2.3. Hematoxylin and eosin (H&E) tissue staining

For the staining procedure, the tissue sections (previously washed in 100% isopropanol) were further washed at room temperature in 80% and 60% isopropanol for 2 min each. The tissues