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Comprehensive proteome analysis of fresh frozen and optimal cutting temperature (OCT) embedded primary non-small cell lung carcinoma by LC–MS/MS

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ABSTRACT

Clinical tissue samples provide valuable information for understanding human diseases. One major type of clinical tissue sample that is amenable to various kinds of analysis is fresh frozen and optimal cutting temperature (OCT)-embedded primary patient tissue. Recent advances in mass spectrometry (MS) technologies have been widely applied to study human proteomes by using clinical specimens. However, polymeric compounds such as OCT can interfere with MS analyses. Here we present methods that enable the preparation and analysis of fresh frozen and OCT embedded primary tissue samples by LC–MS/MS. A scraping method was first introduced to reduce the heterogeneity of OCT-embedded non-small cell lung carcinoma tumor sections. OCT compound was reproducibly removed by a series of washing steps involving ethanol and water prior to trypsin digestion. In data-dependent acquisition mode, optimized dynamic exclusion duration settings were established to maximize peptide identifications. These sample preparation conditions and MS parameter settings should be utilized or carefully adjusted in order to achieve optimal comprehensive proteome characterization starting from fresh frozen and OCT embedded clinical tissue specimens.

1. Introduction

Measuring specific protein expression levels in clinical samples can provide valuable information for disease diagnosis, prevention, and intervention [1]. Immunohistochemistry (IHC) is a method routinely used by clinical pathologists to semi-quantitatively assess protein expression in patient specimens in order to facilitate diagnosis and classification of diseases. However IHC is a subjective method to some extent, depends on access to consistent, high-specificity antibody preparations, and generally lacks high-throughput capability [2]. Recent advances in mass spectrometry (MS) technology and MS-based quantitative proteomics approaches have enabled highly accurate and sensitive targeted and comprehensive protein identification and quantification [3]. These methods have been used to analyze various biological materials, including primary tissue explants, cell lines derived from human tumors, fresh and frozen primary patient tissue, formalin-fixed paraffin-embedded tissues, and bodily fluids such as serum/plasma, cerebral spinal fluid, urine, saliva, pleural effusion, etc.

Established human cell lines are a rich source for proteomics studies. Cultured cells are generally a homogenous population, easily stored and disseminated between laboratories, easy to manipulate, and capable of being adapted for high-throughput analyses and screening. They represent versatile systems with which to study cellular phenotypes and proteome changes associated with experimentally controlled conditions, such as with or without drug treatment, modulated protein expression (e.g., ectopic or RNAi-mediated knock-down), and are amenable to

Abbreviations: OCT, optimal cutting temperature; MS, mass spectrometry; IHC, immunohistochemistry; NSCLC, non-small cell lung cancer; FFPE, formalin-fixed paraffin-embedded; PVA, polyvinyl alcohol; PEG, polyethylene glycol; LCM, laser capture microdissection; LC, liquid chromatography; NOD-SCID, nonobese diabetic severely combined immune deficient; H&E, hematoxylin and eosin; MALDI, matrix-assisted laser desorption/ionization; GO, Gene Ontology; FASP, filter-aided sample preparation; DE, dynamic exclusion.

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metabolic labeling methods to facilitate quantification. However, the in vitro setting is inevitably different from the in vivo environment, and human patient-derived primary tumor xenograft models of human cancers are increasingly available. Therefore, optimized methods are needed for the characterization of primary tissue and related animal models.

Primary patient tissue, suitably preserved and prepared, is a most desirable material for proteome analysis, and is readily available in the case of certain cancers, such as early stage non-small cell lung carcinoma (NSCLC), where surgical resection is often indicated. Moreover, patient-matched "normal" tissue is often collected, which facilitates paired analyses that can reveal dysregulated features (e.g., altered gene expression and methylation levels, and changes in protein expression and post-translational modifications) that are disease associated. Generally there are two common types of clinical specimens, which differ according to how they are preserved: fresh frozen, and formalin-fixed paraffin-embedded (FFPE). Fixing clinical specimens in formalin and then embedding in paraffin is a common procedure to preserve clinical samples at room temperature for long term storage. Various approaches have been developed that enable MS-based proteome analysis of liquefied FFPE samples (reviewed in Ref. [4]), and including, for example, comprehensive proteome analysis [5], and quantification of specific proteins and protein phosphorylations [6].

Fresh or freshly frozen tissues are ideal materials for proteomics analysis, requiring fewer processing steps than FFPE methods. Typically a small piece of tissue is homogenized directly in a detergent-containing lysis buffer in order to disrupt the tissue structure and extract proteins. If proteome sequence, post-translational modifications, and abundance information are under investigation, then denaturing conditions involving, for example, high concentrations of protein denaturing agents such as urea or sodium dodecyl sulfate (SDS) are often used as part of the initial extraction step. However, often tissues are collected with an aim to conduct imaging analyses that require preservation of tissue ultra-structural details. A common approach that, unlike the FFPE method, does not involve covalent cross-linking of tissue protein, is to embed tissue in optimal cutting temperature (OCT) compound. OCT is a cryopreservative medium composed of polyvinyl alcohol (PVA), polyethylene glycol (PEG), and non-reactive ingredients, providing a support matrix for cryostat sectioning. OCT-embedded tissues are typically cut into 4–12-μm-thick sections to facilitate a combination of analyses including histopathological examination and/or laser capture microdissection (LCM). However, the polymers in OCT medium (i.e., PVA and PEG) interfere with MS analysis by suppressing ion formation [7], and thus need to be removed from samples. A few proteomic studies have been conducted in OCT-embedded tissues [8–12]. In some studies, OCT was cleared in subsequent experimental steps (e.g., any separation approach that involved running polyacrylamide gels) [8,9,12]. While in other studies, the number of proteins identified was compromised due to incomplete elimination of OCT medium [10,11]. One challenge associated with fresh frozen tissues is that sample size/amount may be limiting and finite. Another challenge is the heterogeneity of human tissues, which consist of various populations/types of cells. LCM is a widely used technique to isolate certain cell populations from heterogeneous specimens. For example, LCM can be used to separate tumor cells from surrounding stroma and infiltrating immune cells. However, LCM is a laborious procedure when adapted for MS analysis, since several batches of cells must be isolated and pooled in order to obtain sufficient cells of a pure population for proteomics experiments.

Recent developments in MS technologies have provided improved accurate mass measurements, higher scanning speeds, and increased sensitivity and resolution [13]. These technical gains have fostered new MS applications for translational research. Liquid chromatography coupled to tandem MS (LC–MS/MS) is a common approach to analyze complex proteomes.

In this report, we describe details of an optimized workflow to prepare OCT embedded primary tissues for LC–MS/MS analysis, involving enrichment of tumor cells in NSCLC primary tissue sections by dissection of unstained OCT embedded primary tissue, a procedure that completely removes OCT before tissue lysis, and appropriate MS setting optimizations. This protocol has the potential to be generally applicable for the quantitative characterization of primary tissue proteomes.

2. Materials and methods

2.1. Sample collection

H1581 cells were implanted subcutaneously into nonobese diabetic severely combined immune deficient (NOD-SCID) mice to establish xenograft models. The animal study was approved by the University Health Network Human Research Ethics and Animal Care Committee. Once xenografts reached a volume of 1.5 cm^3, the mice were sacrificed and the tumors were immediately excised and cut into approximately 5 mm × 5 mm × 5 mm pieces and stored in liquid nitrogen. One piece of the xenograft tumors and a specimen from a NSCLC patient diagnosed with squamous cell carcinoma (used to generate the histology image shown in Fig. 2) were embedded in OCT compound (Sakura) and were cut into 8 μm serial sections at −21 °C by using a cryostat (Leica CM1950). Sections were mounted on glass slides (Thermo Fisher Scientific) and kept at −80 °C until use. To enrich for tumor cells, a frozen primary tissue section was stained with hematoxylin and eosin (H&E), and then the surrounding non-tumor tissues were scraped away by using a stainless steel surgical blade (size #22; feather). This first, H&E-stained section served as a guide for the removal of non-tumor material in the next 4 consecutive sections; performed on dry ice. The tumor-enriched materials were stored at −80 °C.

HEK293 cells were grown in Dulbecco's modified Eagle medium (SIGMA) supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin–streptomycin. These cells were used as a source of protein for MS parameter optimization experiments.

2.2. Sample preparation

OCT-embedded xenograft tissue sections were used for OCT-removal experiments. Four tumor-enriched tissue sections were scraped into a 1.5 mL tube, and 1 mL ice-cold 70% (v/v) ethanol in HPLC-grade water was added into the tube and gently rotated in a 4 °C refrigerator for 1 min, and centrifuged at 4 °C for 2 min (5000×g). Supernatant was carefully removed and above steps

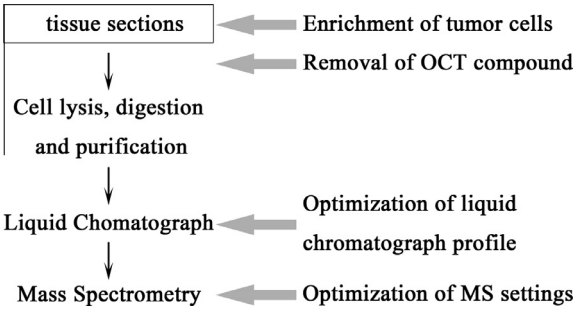

Fig. 1. Overview of the proteomics workflow for analysis of fresh frozen and OCT embedded samples. Grey arrows show the experimental steps that were considered for optimization.