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Decoding the Proteome in Formalin-Fixed Paraffin-Embedded (FFPE) Tissues

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Introduction

Human tissue samples provide ideal subject material for proteomic studies investigating the molecular features of diseases. Formalin-fixed, paraffin-embedded (FFPE) and fresh-frozen tissues are routinely prepared from diagnostic biopsies by clinicians and are often archived, particularly in the case of fixed samples. These specimens are a valuable resource for biomarker discovery because they embody the actual disease in question and harbor all the variations and permutations resulting from disease heterogeneity, diversity among demographic groups, and are often associated with patient records which can provide valuable insights into prognosis and treatment response. In contrast, immortal human cell lines (the dominate laboratory model for many diseases, including cancer) represent a single cell type isolated from one individual and passaged under laboratory conditions, sometimes for decades. These lines can become unknowingly cross-contaminated by other cell lines, may lose important features in the course of culturing and/or may acquire characteristics not present in the original donor tissue. Cell lines have traditionally been more amiable to proteomic analysis than fixed tissues; mass spectrometric techniques such as SILAC, where "heavy" amino acids are introduced to cell culture media, enable quantitative measurements of protein abundance and haveserved as the foundation of proteomic studies. However, fixed or frozen human tissues cannot be grown in the presence of labeled amino acids and during the fixation process amino acid residues necessary for other types of isotopic labeling become masked. These facts, and other challenges, such as variation in fixation time, incomplete or partial fixation of tissue, and degradation of proteins due to long-term storage, havecomplicated proteome analysis of FFPE tissues in the past. Recently, improved protein extraction methods, a better understanding of the chemistry that occurs during fixation and the development of label-free quantitative mass spectrometric techniques have allowed proteome analysis of FFPE samples ranging across a variety of human diseases. The contributions these analyses have made to the diagnosis and treatment of many human diseases is evidence that the field of FFPE proteome analysis has matured beyond its infancy.

Accomplishments in FFPE Proteome Analysis

Profiling of FFPE proteomes has resulted in the discovery of putative biomarkers for several diseases, yielding protein signatures that may improve diagnostic differentiation and could contribute to therapeutic approaches. Proteomic analysis of large cell neuroendocrine carcinoma (LCNEC) and small cell lung carcinoma (SCLC) revealed four candidate biomarkers whose expression differs between the two tissue types [1]. These features could aid in diagnostic differentiation of these two carcinomas, in which pre-therapeutic histological distinction has been problematic. In another example, protein extracts from normal pancreas, chronic pancreatitis and pancreatic cancer FFPE tissue identified exclusively expressed proteins associated with each of the three tissue types [2]. Following further validation, expression of these proteins in pancreatic tissue could improve diagnosis of individuals with diverse pancreatic histologies. Another study of pancreatic cancer, where the proteomes of FFPE pancreatic ductal adenocarcinoma and matched lymph node metastases were analyzed, resulted in the identification of two potential epithelial-specific therapeutic targets, 14-3-3 sigma and S100P [3]. In yet another example, proteome analysis utilizing benign prostate hyperplasia and prostate cancer FFPE tissues identified known prostate cancer markers such as prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP) [4]. This study also compiled a panel of candidate biomarkers, including MIC1, which may prove useful in distinguishing between benign prostate hyperplasia and prostate cancer. A study of breast cancer at defined clinical disease stages revealed potential markers indicative of stage and recurrence [5]. Several others FFPE proteome studies have been performed, resulting in the discovery of putative biomarkers for esophageal adenocarcinoma [6], lung neuroendocrine tumors [7], colorectal cancer [8], tubo-ovarian cancer [9], breast and prostate metastases [10] and cutaneous and metastatic melanoma [11-13]. Other proteomic studies in FFPE malignant melanoma and benign nevi tissues identified potential biomarkers differentiating Spitz nevi from Spitzoid malignant melanoma [14], which can be extremely difficult to differentiate based on histological criteria. In another study, proteomic alterations resulting in activation of hallmark cancer pathways in malignant melanoma relative to benign nevi were discovered, resulting in the identification of new candidate biomarkers as well as potential therapeutic targets [15]. Additionally, a study examining the effects of ionizing radiation on FFPE heart tissue revealed alterations in lipid and pyruvate metabolism as well as mitochondrial impairment [16]. These studies illustrate the value of proteome analysis of fixed tissues and highlight contributions made not only to biomarker discovery but also to therapeutic decision making and to novel drug design.

Challenges of FFPE Proteome Analysis

One of the biggest challenges hindering proteome analysis of FFPE tissues is the efficient extraction of proteins from these samples [17-19]. Since the first report of efficient protein extraction from FFPE tissues for molecular analysis [20] researchers have been adjusting the

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components of protein extraction buffer to yield larger data sets for analysis. Inclusion of at least 2% SDS seems to have a profound effect on fixed cell lysis and protein denaturation [13,15,17,21-27], although it is important to dilute this percentage down before trypsinization because of adverse effects on trypsin activity. Extraction buffer with an alkaline pH (8.0-10.0) has been shown to be optimal for protein extraction [28,29]; buffers with a neutral pH (7.5) or acidic pH (4.0) are less efficient [25,29]. In addition to optimization of extraction buffer, protein yield can be improved through the reversal of crosslinks formed during fixation. Several approaches have been employed for crosslink reversal including heating at 90°C for one hour followed by heating at 65°C for four hours [15]. Heating tissue samples likely contributes to protein extraction through the hydrolysis of intra- and intermolecular methylene bridges (crosslinks) [30,31], which may also open otherwise concealed cleavage sites for trypsin. Several commercially available protein prep kits are currently available, though many groups appear to prepare their own extraction buffers and optimized extraction protocols. In addition to the approaches listed above, other modifications have been made for protein extraction including the use of sonicationto fragment genomic DNA and other nucleic acid components of fixed tissues [15]. These advancements in protein extraction have contributed to the achievements listed above in FFPE proteome research. In the future, cataloging the proteomes of additional diseases as well as annotating prospective biomarkers will be necessary for the realization of the full potential of archival fixed tissues, namely the routine clinical usage of proteomic features of human tissues during diagnosis, prognosis and individualized therapeutic intervention.

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