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TECHNICAL ADVANCE

Application of Selected Reaction Monitoring for Multiplex Quantification of Clinically Validated Biomarkers in Formalin-Fixed, Paraffin-Embedded Tumor Tissue

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One of the critical gaps in the clinical diagnostic space is the lack of quantitative proteomic methods for use on formalin-fixed, paraffin-embedded (FFPE) tissue. Herein, we describe the development of a quantitative, multiplexed, mass spectrometry–based selected reaction monitoring (SRM) assay for four therapeutically important targets: epidermal growth factor receptor, human EGF receptor (HER)-2, HER3, and insulin-like growth factor-1 receptor. These assays were developed using the Liquid Tissue–SRM technology platform, in which FFPE tumor tissues were microdissected, completely solubilized, and then subjected to multiplexed quantitation by SRM mass spectrometry. The assays were preclinically validated by comparing Liquid Tissue–SRM quantitation of FFPE cell lines with enzyme-linked immunosorbent assay/electrochemiluminescence quantitation of fresh cells ($R^2 > 0.95$). Clinical performance was assessed on two cohorts of breast cancer tissue: one cohort of 10 samples with a wide range of HER2 expression and a second cohort of 19 HER2 IHC 3+ tissues. These clinical data demonstrate the feasibility of quantitative, multiplexed clinical analysis of proteomic markers in FFPE tissue. Our findings represent a significant advancement in cancer tissue analysis because multiplexed, quantitative analysis of protein targets in FFPE tumor tissue can be tailored to specific oncological indications to provide the following: i) complementary support for anatomical pathological diagnoses, ii) patient stratification to optimize treatment outcomes and identify drug resistance, and iii) support for the clinical development of novel therapies. *(J Mol Diagn 2013, 15: 454–465; http://dx.doi.org/10.1016/j.jmoldx.2013.03.002)*

The aberrant cellular biochemistry that drives tumor growth and cancer progression is mediated largely by sets of proteins organized into complex cell-signaling networks. In the context of cancer, these proteins may be described as oncoproteins because they are part of biochemical pathways that control malignant transformation and permit unregulated cell growth.[1,2] Individual members of these sets of proteins have been the targets of targeted therapies that have been developed since the 1990s. A notable example is the use of a humanized monoclonal antibody, trastuzumab, for treatment of patients with HER2-positive breast cancer. HER2 is a member of the human epidermal growth factor receptor (EGFR) family of transmembrane tyrosine kinase receptors, along with HER3 and HER4.[3] HER family members can drive tumor growth through receptor homodimerization (HER2/HER2), heterodimerization (formation of HER2/HER3 dimers), and receptor transactivation (c-Met activation of EGFR).[4] In each case, these signaling pathways help to promote the proliferation and survival of cancer cells.[3,5–9] Although specifically targeting HER2 inhibits tumor growth and reduces the risk of metastasis,

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trastuzumab resistance is a common therapeutic occurrence in advanced cancers. In most cases, resistance is driven by alterations in the expression of alternate signaling pathways, not through changes in the drug target, although secondary mutations in EGFR have been described.[10]

Diagnostic testing is essential for identifying the subset of patients with cancer who are best suited for targeted therapy. In the instance of trastuzumab, treatment is restricted to those patients who are $HER2^{+}$, as defined by either high immunohistochemical (IHC) staining and/or high copy numbers of the *HER2* gene, assessed using fluorescence *in situ* hybridization. These methods have been useful surrogates for measuring HER2 protein expression and were instrumental in defining a population of patients with breast cancer who respond to trastuzumab treatment.[3,11–13] Current diagnostic methods do not directly quantify the protein targets nor can they allow for simultaneous multiplex analysis of other proteins involved in tumor growth and drug resistance. Methods using RNA analysis to assess or predict protein expression are not considered universally reliable because they do not directly quantify the expression of the proteins.[14] Indeed, a shortcoming in the clinic is the lack of availability of diagnostic platforms and methods that can generate quantitative and reproducible diagnostic information about multiple functional proteins and produce valuable information about which proteins should be targeted for therapeutic intervention. More important, these diagnostic methods must be applicable to formalin-fixed, paraffin-embedded (FFPE) tissue to be seamlessly integrated into clinical practice.

Mass spectrometry (MS) has been used in clinical assays, such as for the assessment of multiple inborn errors of metabolism[15] and vitamin D levels.[16] The ability to accurately measure target analytes from small amounts of biological samples separates MS from most other diagnostic methods. More recently, MS has begun to be used for quantitative analysis of proteins in biological samples,[17] and application of MS to patient-derived FFPE tissue can be expected to have a profound impact on patient stratification and targeted cancer therapeutics.[18–23] The Liquid Tissue—selected reaction monitoring (SRM) work flow (Figure 1) is a proteomic method by which microdissected FFPE tumor tissue is subjected to Liquid Tissue processing to reverse formalin cross-links. This is followed by trypsinization to completely solubilize all of the protein in the sample. This tryptic peptide mixture is then subjected to SRM analysis using stable isotope-labeled control peptides for accurate quantitation. SRM methods have long been used to quantitate low-abundance protein targets in plasma,[24] but application of these techniques to FFPE tissue samples has, until recently, been hindered by incomplete solubilization of samples.[25–33] We have recently described the development and clinical performance of a Liquid Tissue—SRM assay for EGFR in FFPE patient tissues.[29] By using this quantitative EGFR-SRM assay, we assessed EGFR expression in FFPE tumor tissues from a cohort of gefitinib-treated patients with non-small cell lung cancer. Although each of these tumors was positive for EGFR by IHC, by using quantitative SRM, we demonstrated a wide range of EGFR expression in these tissues. Although the focus of the Liquid Tissue—SRM assay development has been on FFPE tissue, recent reports have shown that these MS-based methods can also be performed with equal precision on frozen tissue.[12,34]

This report describes the development of a multiplexed Liquid Tissue—SRM assay to accurately measure the expression of a series of oncological targets: the insulin-like growth factor-1 receptor (IGF-1R and IGF-1R-SRM), HER2 (HER2-SRM), and HER3 (HER3-SRM). We also include the assay development results of EGFR-SRM to place them in the context of this larger multiplexed set.[29] Each of the assays is capable of detecting and measuring attomole (ie, 10^{-18} mol) amounts of these specific peptides directly in FFPE patient tumor tissue. We extend the assay development to assess the performance of these assays (ie, HER2-SRM, EGFR-SRM, and HER3-SRM) in FFPE patient breast tumor tissues, including patients who had been treated previously with trastuzumab. This study demonstrates the broad, dynamic range of SRM MS, when compared with HER2 IHC, underlining the gaps in using only IHC to assess protein expression levels. Taken together, Liquid Tissue—SRM provides a powerful, quantitative, and multiplexed approach for analysis of critical oncological proteins in FFPE tumor tissues.

Materials and Methods

Cell Lines

Ten human cancer cell lines were used: A431 (skin), HCC-827 (lung), MDA-MB-231 (breast), MCF7 (breast), HT29 (colon), Colo205 (colon), T47D (breast), SKBR-3 (breast), PC3 (prostate), and ZR75-30 (breast). A431 and MDA-MB-231 lines were maintained in Dulbecco's modified Eagle's medium; T47D, ZR75-30, and HCC827 cells were maintained in RPMI 1640 medium; MCF7 cells were maintained in Dulbecco's modified Eagle's medium—F12 medium; SKBR-3 and HT29 cells were maintained in McCoy's 5A medium; and PC3 cells were maintained in HAM's-F12 medium. All media were supplemented with 10% fetal bovine serum and antibiotics. Each cell line was grown in sufficient quantity so that cells could be fixed in formalin and embedded in paraffin for SRM analysis, as well as preparing fresh lysates in parallel for analysis by immunoassay [ie, either electrochemiluminescence (ECL) or enzyme-linked immunosorbent assay (ELISA)].

For SRM analysis, the cultured cell lines were prepared by pelleting the cell suspensions, overlaying the pellet with 10% neutral-buffered formalin, and allowing the cells to fix for 18 to 24 hours at 4°C. The 10% formalin was removed, and the pellet was washed with water and then transferred into 70% ethanol. Embedding in paraffin and dividing into sections cells prepared on microscope slides were done using standard histological methods.