DNA Repair 65 (2018) 47–53

Contents lists available at ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair

Targeted mass spectrometry enables robust quantification of FANCD2 mono-ubiquitination in response to DNA damage

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ARTICLE INFO

Keywords:
Fanconi anemia
Multiple reaction monitoring
DNA damage response
Immuno-MRM

ABSTRACT

The Fanconi anemia pathway is an important coordinator of DNA repair pathways and is particularly relevant to repair of DNA inter-strand crosslinks. Central to the pathway is monoubiquitination of FANCD2, requiring the function of multiple proteins in an upstream Fanconi core complex. We present development and analytical characterization of a novel assay for quantification of unmodified and monoubiquitinated FANCD2 proteoforms, based on peptide immunoaffinity enrichment and targeted multiple reaction monitoring mass spectrometry (immuno-MRM). The immuno-MRM assay is analytically characterized using fit-for-purpose method validation. The assay linear range is > 3 orders of magnitude with total repeatability < 16% CV. In proof-of-principle experiments, we demonstrate application of the multiplex assay by quantifying the FANCD2 proteoforms following mitomycin-c treatment in an isogenic pair of *FancA*-corrected and uncorrected cell lines, as well as primary peripheral blood mononuclear cells from Fanconi Anemia patients. Additionally, we demonstrate detection of endogenous FANCD2 monoubiquitination in human breast cancer tissue. The immuno-MRM assay provides a potential functional diagnostic for patients with Fanconi Anemia with defects in the upstream FA complex or FANCD2, and a potential test for predicting sensitivity to DNA cross-linking agents in human cancers.

1. Introduction

Fanconi anemia (FA) is an inherited disorder characterized by progressive bone marrow failure and an increased risk of cancers, especially during early adulthood. FA patients harbor mutations in a set of genes encoding the FA pathway, a network of 21 proteins that is specialized for repairing DNA inter-strand cross-links [1]. Activation of the FA core complex results in the monoubiquitination of FANCD2 and FANCI, which interact with several downstream components (e.g. BRCA1, Rad51, BRCA2) for DNA repair [2]. Cells with mutations in any of the core complex proteins lack the ability to monoubiquitinate FANCD2, making FANCD2 ubiquitination a convergence point in the pathway, with an estimation of > 90% FA patients defective in this step [1,3]. FA patients experience excessive toxicities with chemotherapy or radiation, so early diagnosis is essential to inform therapeutic decisions [4]. Additionally, somatic mutations In FA genes render tumor cells sensitive to DNA crosslinking agents, so identification of FA pathway defects provides an opportunity for therapeutic targeting [5–7].

Diagnosis of FA based on presenting symptoms alone is difficult and unreliable due to the broad phenotypic spectrum of FA and overlapping features with other genetic (and nongenetic) diseases. A significant subset of FA patients lack characteristic physical findings [8]. Currently, the standard for FA diagnostic testing is manual enumeration of chromosomal breakage following exposure to clastogens [9,10]. In this test, T lymphocytes in peripheral blood are stimulated with phytohaemagglutinin (PHA) to proliferate in the presence or absence of DNA cross-linking agents, typically mitomycin C (MMC) or diepoxybutane (DEB), followed by counting chromosomal breaks and fusions in metaphase spreads. Chromosomal breakage may also be assessed in skin fibroblasts, since somatic mosaicism may result in a falsely negative blood test. The approach is laborious and difficult to standardize, partly because results are dependent on several variables (e.g. culture time,

Abbreviations: FA, Fanconi anemia; MRM, multiple reaction monitoring; PTMs, post-translational modifications; MS, mass spectrometry; DDR, DNA damage response; MMC, mitomycin-C; LCL, lymphoblast cell line; WB, Western blot; VUS, variants of undetermined significance; IR, ionizing radiation

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https://doi.org/10.1016/j.dnarep.2018.03.003
Received 6 February 2018; Received in revised form 19 March 2018; Accepted 19 March 2018
Available online 21 March 2018
1568-7864/

exposure time, concentration, threshold for positive/negative). Molecular subtyping by DNA sequencing or complementation analysis is increasingly utilized clinically, though interpretation is complicated by variants of unknown significance [11], ascertainment of biallelic mutation distribution, and distinguishing constitutional versus somatic mutations [12,13].

Since pathogenic mutations in FA genes functioning upstream of the FANCD2 complex impair FANCD2 monoubiquitination, an assay to directly quantify the level of monoubiquitinated FANCD2, and thus determine defects in the pathway, would provide a complementary functional readout to existing assays. However, testing of FANCD2 monoubiquitination with traditional immunoassays or intracellular localization assays are low throughput and semi-quantitative [3,14], establishing a need for a more effective quantitative assay.

Targeted, multiple reaction monitoring mass spectrometry (MRM) is a quantitative approach capable of quantifying analyte peptides with high sensitivity and specificity [15,16]. MRM has been used in clinical laboratories for decades to measure small molecules [17], and is increasingly applied in protein and peptide measurement [18–21]. The *targeted* MRM mode of proteomic analysis is different from untargeted ("shotgun") discovery proteomics, where methods are designed to profile as many peptides as possible by matching MS/MS spectra to protein databases. In contrast, for MRM, protein lysates from biospecimens are proteolyzed (typically with trypsin), and proteotypic peptide sequences *unique to the protein* of interest ("proteotypic peptides") are *targeted* for detection by MRM mass spectrometry (Fig. 1A). Instrument and method parameters are optimized to enable the most sensitive and specific measurement of the targeted peptide as a surrogate for the protein of interest. Furthermore, a synthetic, HPLC-purified, stable isotope-labeled version of the analyte peptide is spiked into the biospecimen at a known concentration as an internal standard. The internal standard peptide serves several functions, including: i) to control for much of the preanalytical and all of the analytical variation in the assay, ii) to enable precise, relative quantification of the endogenous analyte, and iii) to help confirm specificity of the assay. The high specificity of MRM-based assays is based on detection of multiple MRM transitions (i.e. combinations of precursor/fragment ions), the chromatographic co-elution of analyte and internal standard, and the consistency of the relative transition ratios between the endogenous and standard peptides. In addition to being highly specific, MRM is standardizable and transferrable across laboratories [22–25], is capable of highly multiplexed measurements [25,26], and is capable of quantifying cell signaling events through chromatographic enrichment of low abundance analytes (e.g., ubiquitinated or phosphorylated peptides) [27–30]. A public repository of highly characterized targeted MS assays has been created [31], and the assay platform is beginning to gain acceptance in clinical laboratories [32,33]. For example, an MRM-based assay targeting a single proteotypic peptide sequence unique to the human thyroglobulin protein is used by clinical laboratories as a surrogate to quantify thyroglobulin (2768 amino acids, ~300 kDa) in patients that make autoantibodies that interfere with conventional immunoassays [34,35]. Other examples include similar assays developed for apolipoproteins [36] and troponin [37].

MRM-based assays have several advantages over conventional protein measurement technologies (e.g., Western blotting, immunohistochemistry, ELISA), such as direct detection of the analyte by a mass spectrometer (contributing to high specificity), the use of internal standards (enabling reproducibility and inter-laboratory standardization), the capability to readily multiplex analysis of many target peptides, a large linear range (typically $\geq 10^3$), and relatively less time and cost required for assay development [38]. In MRM, cross reactivity of antibodies usually does not affect specificity, because the mass spectrometer directly detects the target of interest, and interferences are detected based on analytical criteria [39]. In contrast, Western blots (WB) can feature multiple nonspecific bands and uncertainties about which band represents the analyte of interest. While WB is semi-quantitative at best and has not been standardized across laboratories, MRM uses HPLC-purified, synthetic, internal stable isotope-labeled standard peptides (spiked into the biospecimen at a known concentration) for standardization across laboratories [22–24].

We recently demonstrated the utility of peptide immuno-MRM (coupling immuno-affinity enrichment of peptides to MRM) to quantify the pharmacodynamics of post-translationally modified (e.g. phosphorylated and ubiquitinated) peptides in response to DNA damage [28,30]. Here, we present a multiplex immuno-MRM assay for quantifying the unmodified and monoubiquitinated proteoforms of a proteotypic peptide unique to FANCD2 (K561). The assay is analytically characterized using fit-for-purpose method validation, including linearity, repeatability, and stability. Proof-of-principle application of the assay is demonstrated using human immortalized cell lines (congenic FancA+/−) and Fanconi Anemia patient primary human T cells treated with the DNA cross-linking agent MMC. We also demonstrate detection of monoubiquitinated FANCD2 in breast tumor tissue. The assay can be multiplexed with other immuno-MRM assays [28,30] to profile more comprehensively the DNA damage response in a variety of sample types and conditions, making it useful for a range of biomedical research studies.

2. Materials and methods

See supplementary material for full materials and methods.

3. Results and discussion

3.1. Method development and parameter optimization

An overview of the workflow for the immuno-MRM assay is shown in Fig. 1A. Development of the assay requires identification of a proteotypic peptide sequence (i.e. unique to the protein of interest) for measurement, generation of affinity reagents for enrichment of the targeted peptide, and optimization of analytical parameters for detection of the endogenous and synthetic peptide standards. To develop an assay, we first identified the target peptide analyte as the tryptic fragment surrounding the K561 site of ubiquitination on FANCD2. A blast search confirmed that this sequence is unique to the FANCD2 protein. Ubiquitination covalently attaches the ubiquityl moiety to the lysine at position 561, blocking the site from enzymatic digestion. Additionally, there is a trypsin cleavage site at the lysine residing on the ubiquityl moiety. Thus, following trypsin digestion, the modified protein produces a peptide encompassing amino acids 561–569 containing a −GG remnant on K561, and the unmodified protein produces a fully tryptic peptide of amino acids 562–569 (see Fig. 1B). We sought to develop an immuno-MRM assay capable of measuring both forms of the peptide (monoubiquitinated and unmodified).

We followed established protocols to generate affinity reagents for use in peptide immunoaffinity enrichment [38,40]. The modified peptide containing the ubiquitin (i.e. –GG) remnant was used as the immunogen sequence in generating an anti-peptide rabbit monoclonal antibody. Using established protocols [40], monoclonal supernatants were screened against both forms of the peptide (modified and unmodified) to obtain a clone that was capable of immuno-precipitating both forms. Purified antibodies were subjected to quality control by BCA assay for protein concentration, SDS gel to confirm intact antibody, and recovery efficiency in immuno-MRM enrichments to confirm antibody activity (data not shown).

Synthetic analyte peptides (i.e., HPLC-purified, heavy stable isotope-labeled versions of the modified and unmodified tryptic peptides) were used to optimize the mass spectrometer parameters [41]. Tandem mass spectrometry was used to analyze the HPLC-purified synthetic peptides to confirm the peptide identity and select the best combinations of precursor and fragment ions (i.e. transitions) to be used for identification and quantification of the peptide during MRM. Following