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Quantitative analysis of wild-type and V600E mutant BRAF proteins in colorectal carcinoma using immunoenrichment and targeted mass spectrometry

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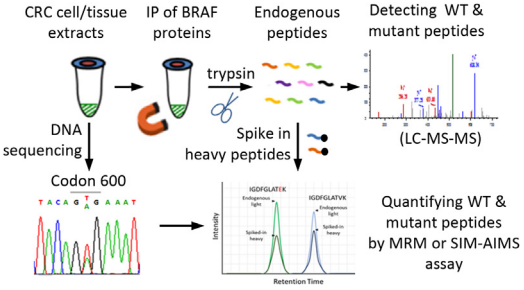
HIGHLIGHTS

- Immunoenrichment coupled with MRM or SIM-AIMS assay was evaluated for quantifying WT and V600E mutant BRAF proteins in CRC.
- Both assays could successfully detect/quantify both targets in a CRC cell line and CRC tissue samples.
- Both targets could be quantified at the level of sub ng/mg protein extract.
- The MRM assay is more sensitive than the SIM-AIMS assay.
- The ratio of V600E mutant/WT BRAF protein was determined in CRC tissue samples.

GRAPHICAL ABSTRACT



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ABSTRACT

The BRAF V600E mutation is one of the most common mutations implicated in the development of several types of cancer including colorectal cancer (CRC), where it is associated with aggressive disease phenotypes and poor outcomes. The status of the BRAF V600E mutation is frequently determined by direct DNA sequencing. However, no previous study has sought to quantify the BRAF V600E protein in cancer specimens. Here, we evaluated immunoenrichment coupled with two MS-based quantitative techniques, namely multiple reaction monitoring (MRM) and single ion monitoring conjugated accurate inclusion mass screening (SIM-AIMS), to detect and precisely quantify wild-type (WT) and V600E mutant

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BRAF proteins in DNA sequence-confirmed CRC tissue specimens. WT and V600E BRAF proteins were immunoprecipitated from a CRC cell line (HT-29), and their representative peptides (^{592}IGDFGLATVK^{601} and ^{592}IGDFGLATEK^{601}, respectively) were confirmed by LC-MS/MS analysis and then quantified by MRM or SIM-AIMS with spiked stable isotope-labeled peptide standards. Both assays worked well for measuring WT BRAF from different amounts of HT-29 cell lysates, but the MRM assay was more sensitive than SIM-AIMS assay for quantifying lower levels of V600E BRAF. In protein extracts (2 mg) from 11 CRC tissue specimens, the MRM assay could measure WT BRAF in all 11 cases (0.32–1.66 ng) and the V600E BRAF in two cases (0.1–0.13 ng; mutant-to-WT ratio, 0.16–0.17). The SIM-AIMS assay could also detect WT and V600E BRAF in CRC specimens, but the measured levels of both targets were lower than those determined by MRM assay. Collectively, this study provides an effective method to precisely quantify WT and V600E BRAF proteins in complex biological samples using immunoenrichment-coupled targeted MS. Since the V600E BRAF protein has emerged as an important therapeutic target for cancer, the developed assay should facilitate future BRAF-related basic and clinical studies.

1. Introduction

In recent decades, millions of somatic mutations have been found in human cancers [1]. Among them, a subset known as "driver mutations" confer selective growth advantages and have been implicated in cancer development [2–4]. These mutations, which occur in what are termed "driver genes," can change the structure of an encoded protein to trigger aberrant activity. Therefore, mutant proteins encoded by driver genes are frequently used as predictive markers and/or molecular targets in cancer therapy [5,6].

BRAF (v-raf murine sarcoma viral oncogene homolog B1) is a member of the RAF kinase family and a key component of the Raf/MEK/ERK (MAPK) signaling pathway. Upon activation by RAS, BRAF phosphorylates its downstream effectors, the MEK kinases, to regulate cell biological activities [7,8]. The BRAF V600E mutation accounts for more than 90% of the BRAF mutations identified in melanoma, papillary thyroid and colorectal cancer (CRC). This mutation can trigger a 500-fold increase in the kinase activity of BRAF, constitutively activating the MAPK signaling pathway which is responsible for tumor initiation and/or development [9–12].

The BRAF V600E mutation occurs in 10–15% of CRC and is frequently associated with genome-wide CpG island hypermethylation and mismatch-repair deficiencies [10–15]. A recent analysis of The Cancer Genome Atlas (TCGA) database revealed that BRAF-mutated tumors also tend to have multiple mutations in Wnt pathway-associated genes [1]. The accumulation of mutations involving the Wnt-APC-β-catenin signaling pathway is known to be a key step in the initiation and early progression of CRC [16]. In addition, EGFR-directed therapy has proven to be ineffective in CRC patients with BRAF mutation [17,18]. A growing body of evidence indicates that BRAF-mutated tumors have poor clinical outcomes [19,20]. Based on this, researchers have sought to combine BRAF (V600E)-specific inhibitor and PI3K inhibitor treatments as a novel targeted therapy in clinical trials for BRAF-mutant CRC patients [21]. Together, the previous studies show that the mutant BRAF protein could be useful as a therapeutic target and prognostic marker for CRC.

Currently, the BRAF mutation status is assessed at the DNA level, rather than by examination of the encoded protein [22]. However, genomic abnormalities are not totally consistent with cancer phenotypes [23], making it important to measure the levels of mutant proteins in cancer. Proteins are traditionally detected by antibody-based methods, including the widely used Western blot and ELISA approaches. However, it has proven difficult to develop a specific antibody for each mutant epitope in general. An existing BRAF V600E-specific antibody (VE1) can be used to detect BRAF V600E mutated proteins in formalin-fixed, paraffin-embedded (FFPE) tissue samples. However, this is considered to be a qualitative, rather than precisely quantitative method [24]. In addition, the BRAF mutant protein is typically expressed at quantities too low to be easily quantified in clinical samples. Thus, we need a highly sensitive technique that can quantify BRAF V600E mutant proteins in complex biological samples.

Multiple reaction monitoring-mass spectrometry (MRM-MS) operated in triple quadrupole mass spectrometer has been widely used to quantify target proteins by measuring peptides generated by proteolytic treatment [25–29]. Conventional MS experiments scan a broad mass-to-charge ratio (m/z) range to characterize proteins in cell extracts. In the MRM assay, in contrast, at least three peptide-specific transitions are scanned across narrow m/z ranges, greatly enhancing the specificity and sensitivity of this technique. MRM-based quantification can detect proteins in the fmol to amol range, and thus provides sufficient sensitivity for the measurement of low-abundance protein variants in complex biological samples [30,31]. Furthermore, the use of stable isotope-labeled standards (SISs) as internal controls can improve the confidence of the absolute quantifications obtained from MRM assays [32].

In addition to MRM, several targeted proteomic techniques have been developed for accurate protein quantification. Recently, the single ion monitoring (SIM) in MS mode and parallel reaction monitoring (PRM) in MS/MS mode have been applied in a new configuration of quadrupole-orbitrap mass spectrometer, which can provide high resolution and accurate mass for targeted proteomic quantification [33]. The SIM method operated on linear ion trap-orbitrap mass spectrometer or triple quadrupole mass spectrometer has been reported to successfully quantify protein or small molecule based on the measurement of precursor ion [34,35]. Another method called accurate inclusion mass screening (AIMS) operated on the orbitrap MS system selectively acquired the MS/MS spectrum against masses on the inclusion list for sequence confirmation of specific targets [36]. Therefore, it is conceivable that the combined use of SIM and AIMS techniques on the linear ion trap-orbitrap mass spectrometer may represent an alternative approach to the MRM assay for quantification of specific targets in complex biological samples.

In this work, we report the evaluation and successful development of immunoenrichment-coupled targeted mass spectrometry methods, including MRM (using triple quadrupole mass spectrometer) and SIM-AIMS (using linear ion trap-orbitrap mass spectrometer), to detect and quantify both wild-type (WT) and V600E mutant BRAF proteins in a CRC cell line and CRC tissue samples. This is the first report to show the accurate quantification of BRAF V600E mutant proteins in complex biological samples.