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Targeted data-independent acquisition for mass spectrometric detection of RAS mutations in formalin-fixed, paraffin-embedded tumor biopsies

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ABSTRACT

Genomic testing for KRAS and NRAS mutations in clinical biopsies of various cancers is routinely performed to predict futility of anti-epidermal growth factor receptor (anti-EGFR) therapies. We hypothesized that RAS mutations could be detected and quantified at the protein level for diagnostic purposes using data-independent acquisition (DIA)-based mass spectrometry in formalin-fixed, paraffin-embedded (FFPE) tumor samples. We developed a targeted DIA assay that surveys the specific mass range of all possible peptides harboring activating mutations in KRAS exon 2. When the assay was applied to tumor samples with known KRAS or NRAS mutations (G12A, G12D, G12V, and G13D), RAS-mutant and wild-type peptides were successfully detected in 11 of 13 biopsy samples. Mutation statuses obtained by DIA were concordant with those obtained by DNA sequencing, and yields of mutant peptide (mutant peptide/[mutant + wild-type peptides]) exhibited linear correlation with yields of RAS-mutant mRNA. When applied to biopsy samples with failed DNA testing results, the DIA assay identified an additional RAS-mutated sample.

Significance: Proteomic detection of RAS mutations by DIA in tumor biopsies can provide solid evidence of mutant RAS protein regardless of the mutation types and sites in exon 2. This robust method could rescue samples that fail genomic testing due to insufficient tumor tissue or lack of sequenceable DNA. It can be used to explore the relationship between protein expression level of mutant RAS and therapeutic outcome.

1. Introduction

Kirsten rat sarcoma viral oncogene homolog (KRAS) is a GTPase transductor protein involved in cell growth and proliferation. It is encoded by KRAS, a well-known proto-oncogene whose permanent activation through mutation is common in human cancers. Sequence alteration of the KRAS gene can impair the ability of KRAS protein to hydrolyze GTP, thus rendering KRAS protein constitutively active. Consequent down-stream activation leads to tumorigenesis. Activating mutations at the GTP-binding site of KRAS occurs in exon 2 (codons 12 and 13), exon 3 (codon 61), and exon 4 (codon 146), with exon 2 harboring > 95% of activating mutations [1]. NRAS, one of the RAS sub-family, has highly homologous sequence with KRAS and its mutation status is also used to select patients for EGFR antibody therapies [2–4]. Mutations in HRAS, the third member of the RAS family, occur infrequently and the mutations in RAS family members are mutually exclusive suggesting a functional redundancy [5].

In the clinic, RAS mutation testing is a reliable way to predict resistance to EGFR-inhibiting therapies; patients with RAS-mutant metastatic colorectal cancer (CRC) are advised to forgo anti-EGFR therapies such as cetuximab and panitumumab [6,7]. RAS mutational status is also used to select non-small-cell-lung cancer (NSCLC) patients for treatment with mitogen-activated protein kinase inhibitor therapy [8,9]. The Food and Drug Administration has approved several in vitro KRAS diagnostic kits that detect nucleic acid of nonsynonymous single nucleotide substitutions [10–14]. DNA-based RAS analysis is effective and useful for informing therapeutic decisions, however there are several opportunities for protein-based RAS analysis as a complimentary method. For example, biopsies from colon and lung cancers can be extremely small, yielding insufficient amounts of tumor tissue or sequenceable DNA. Biopsies of neoplastic bone disease are typically subjected to acid-based decalcification agents prior to sectioning and molecular analysis; while decalcification can degrade or destroy DNA, it does not have deleterious effects on protein. In addition, protein-based RAS mutational analysis can be used for verifying potential neoantigens at the protein level. Recently, a cancer patient harboring G12D KRAS mutation responded to treatment with autologous T cells specific for G12D mutant after identification of T cell-activating neoantigens [15]. Finally, it is possible that absolute or relative levels of RAS mutant protein expressed in tumor tissue could predict therapeutic response or

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resistance, but this has not been explored.

Mass spectrometry (MS) has emerged as a promising technology in clinical oncology, providing multiplexed quantitative analysis of protein biomarkers in biopsied tumor tissues [16–19]. MS-based molecular analysis objectively quantifies protein [20] and is not affected by conformational change which can bias antibody-based assays [21]. Proteomic studies of KRAS mutant protein have typically relied on parallel reaction monitoring (PRM) or selected reaction monitoring (SRM) assays designed to detect tryptic peptides harboring known mutations using synthesized stable-isotope labeled (SIL) peptides spiked-in to the samples as internal standards [22–24]. Such methods have been applied to cell lines, extracellular vesicles, and frozen tissues. To be useful in precision medicine, a KRAS mutation test must be applicable in small FFPE biopsies and where results of genomic testing may not be available.

In the present study, we developed a novel MS-based assay to detect all possible KRAS exon 2 mutations in codons 12 and 13, which account for > 95% of KRAS mutations [1]. We used a peptide-centric targeted DIA approach, [25,26] constructing a high-quality spectral library of target peptides, and querying DIA data to verify the presence of target peptides. Following evaluation of the assay in FFPE tumor biopsy samples harboring known KRAS or NRAS mutations (G12D, G12A, G12V, and G13D), the assay was applied to biopsy samples with failed DNA testing results. We also compared RAS mutation yields at the protein level to RAS mutation yields at the mRNA level.

2. Materials and methods

2.1. Tissue samples

Slides of FFPE tumor tissue were received in the authors' clinical laboratory during 2017. Patients provided consent for research use of anonymized data from their test results.

2.2. Protein extraction and peptide generation

Peptides for proteomic analysis from the tissue slides were prepared as previously described [27,28]. Briefly, tissue sections mounted on DIRECTOR® slides (Expression Pathology, Rockville, MD) were deparaffinized, stained with hematoxylin, and subjected to microdissection after tumor-specific markup by a board-certified pathologist. Microdissection was performed on an MMI (Eching, Germany) laser microdissection system. Collected cells were heated in Liquid Tissue® buffer (Expression Pathology, Rockville, MD) at 95 °C, and incubated with trypsin (Promega, Madison, WI) for 16 h at 37 °C. A Micro BCA assay (Thermo Fisher Scientific, Rockford, IL) was used to determine total protein concentration. Peptide mixture equivalent to 2 μg total protein was used for each analysis. iRT peptide mix (Biognosys AG, Schlieren, Switzerland) was spiked in each sample before LC-MS to calibrate retention times.

2.3. Synthetic peptides and spectral library

Synthetic peptides were purchased from Thermo Fisher Scientific (Rockfort, IL) including four RAS mutant peptides (G12A: LVVVGAAGVGK, G12D: LVVVGADGVGK, G12V: LVVVGAVGVGK, G13D LVVVGAGDVGK) and a wild-type peptide (LVVVGAGGVGK). A peptide mixture was prepared (50 fmol/μL) in 0.1% formic acid solution and analyzed in data-dependent acquisition (DDA) mode using a Q-Exactive HF mass spectrometer (Thermo Fisher Scientic, Bremen, Germany). Of note, spectra for G12D and G13D peptides were acquired using individual LC-MS analyses because these peptides are isobaric and co-elute in this LC method. A spectral library was built with the resulting DDA data using Spectronaut™ Pulsar 11.0 (Biognosys, Schlieren, Switzerland), and this library was incorporated into an existing library built in-house from multiple samples for general proteomics studies. The Pulsar search for library generation used the following parameters: digest rule = trypsin/P, toggle N-terminal M = yes, variable modifications = acetyl (protein N-term), deamidation (NQ), Gln- > pyro-Glu, oxidation (M). The library generation parameters are as follows: search engine = Pulsar, PSM false discovery rate (FDR) cutoff = 1%, protein FDR cutoff = 1%, digestion rule = trypsin/P, N fragment per peptide = 4–8, minimum AA length = 3, minimum relative intensity = 5. All data was searched against the UniProtKB/Swiss-Prot *H. sapiens* canonical sequence database (version 25 Oct 2017) [29]. For the synthetic mutant peptides, the Uniprot database was modified by adding additional sequences of mutant peptides.

2.4. LC-MS analysis

All LC-MS analyses were performed with an Ultimate 3000 UHPLC system coupled to a Q- Exactive HF mass spectrometer. Samples were loaded in 1% formic acid onto the trap column (Acclaim PepMap, 0.10 mm × 20 mm, 5 μm C18 particles) at a flowrate of 4 μL/min for 10 min. Samples were then separated by the analytical column (Acclaim PepMap, 0.75 mm × 250 mm, 2 μm C18 particles) at a flowrate of 300 nL/min. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The complete mobile phase gradient was composed of the following steps (time in minutes, % B): 0, 1%; 10, 1%; 15, 6%; 90, 24%; 100, 36%; 100.1, 90%; 107, 90%, 113, 1%; 120, 1%. Both the trap column and analytical columns were held at 50 °C using a column heater. The column eluent was delivered to the Q-Exactive HF using nano-electrospray. The electrospray voltage of 1.9 kV was applied to a metal emitter (Thermo ES542). For all DIA analyses, the MS1 scan covered precursors between 400 and 1200 *m/z* at 60 k resolution. MS2 scans acquired with a precursor isolation window of 2 Th, HCD fragmentation (normalized collision energy = 27) and at 30 k resolution. A total of 45 MS2 windows were used to cover a range from 470 to 560 *m/z*. Additional mass spectrometry parameters are as follows: MS1 AGC target = 3e6, MS1 max IT = 20 ms, MS2 AGC target = 3e6, MS2 max IT = auto.

2.5. DIA data analysis for relative quantification

Targeted DIA data analysis was performed using Spectronaut™ Pulsar 11.0 (Biognosys, Schlieren, Switzerland) software, which analyzes the extracted ion chromatograms (XICs) of product ions against a spectral library. The FDR was estimated with the mProphet [30] approach and set to 1% at peptide precursor level and at 1% at protein level. Quantification is conducted by summing area under the curve (AUC) values of all identified fragments minus the AUC of the fragments flagged for interference by the software. Additional parameters are following: data extraction = dynamic, XIC extraction = dynamic, calibration = automatic, decoy method = scrambled, precursor Qvalue cut off = 0.01, protein Qvalue cut off – 0.01, Pvalue estimator = kernel density estimator, interference correction = yes, Quantity MS-level = MS2, Quantity type = area, data filtering = Qvalue, cross run normalization = Qvalue sparse and local normalization.

3. Results and discussion

3.1. Assay development

A DIA-based assay capable of detecting all possible mutations (including unreported mutations) in KRAS exon 2 was designed for use in samples with an unknown genetic mutation status. Table 1 summarizes the combination of possible genetic changes resulting single nucleotide substitution of codons 12 and 13 of KRAS gene and their translations at the protein level. The 11-mer peptides shown in this table are the expected products from trypsin and/or Lys-C digestion (G12R and G13R products can be generated from Lys-C digestion only). Their predicted *m/z* values of doubly-charged peptide ions range from 478.2907 to

Table 1
Possible peptides harboring activating mutations of KRAS in exon 2.

		Codon	AA	Peptides	Type	Mw	$[M + 2H]^{2+}$
12Gly	WT	G G T	Gly	LVVVGAGGVGK	WT	954.5815	478.2907
	Mutation	G A T	Asp	LVVVGADGVGK	G12D	1012.5869	507.2935
		G C T	Ala	LVVVGAAGVGK	G12A	968.5971	485.2986
		G T T	Val	LVVVGAVGVGK	G12V	996.6284	499.3142
		A G T	Ser	LVVVGASGVGK	G12S	984.5920	493.2960
		C G T	Arg	LVVVGARGVGK	G12R	1053.6611	527.8306
		T G T	Cys	LVVVGACGVGK	G12C	1000.5692	501.2846
13Gly	WT	G G C	Gly	LVVVGAGGVGK	WT	954.5815	478.2907
	Mutation	G A C	Asp	LVVVGAGDVGK	G13D	1012.5869	507.2935
		G C C	Ala	LVVVGAGAVGK	G13A	968.5971	485.2986
		G T C	Val	LVVVGAGVVGK	G13V	996.6284	499.3142
		A G C	Ser	LVVVGAGSVGK	G13S	984.5920	493.2960
		C G C	Arg	LVVVGAGRVGK	G13R	1053.6611	527.8306
		T G C	Cys	LVVVGAGCVGK	G13C	1000.5692	501.2846

527.8307. We hypothesized that a DIA approach with a gas-phase fraction within a specified *m/z* range would detect the peptides harboring any activating mutation in KRAS exon 2 with a single nucleotide substitution, as well as wild-type peptides.

For evaluation of the method designed to detect multiple types of exon 2 mutations, we selected four KRAS mutations (G12D, G12A, G12V, and G13D) of high prevalence in many cancers, and particularly in CRC [1,31,32]. A spectral library was built using five synthetic peptides representing these four mutant variants of KRAS protein plus wild type. LC-MS attributes of the synthetic peptides as well as the prominent product ions generated from each peptide were acquired (Table 2).

Mutations in codons 12 and 13 generate a set of isobaric peptides due to the same composition of amino acids (Table 1). We compared the LC-MS characteristics of the isobaric peptides using the peptide harboring G12D, the most frequent mutation at exon 2. The isobaric peptides LVVVGADGVGK (G12D) and LVVVGAGDVGK (G13D) have an identical sequence except for two amino acids transposed in the center.

Table 2
LC-MS attributes of KRAS peptides used in the spectral library.

Peptides		Precursors		Product ions		
Mutation	Sequence	$[M + 2H]^{2+}$	iRT[a]	Ion	m/z	Rel. Int
WT	LVVVGAGGVGK	478.2907	16.3	y7	545.3042	1.00
				y9	743.4410	0.69
				y8	644.3726	0.60
				b3	312.2282	0.29
				y5	417.2456	0.24
G12A	LVVVGAAGVGK	485.2986	19.3	y7	559.3198	1.00
				y9	757.4567	0.72
				y8	658.3882	0.62
				y4	360.2242	0.33
				b3	312.2282	0.32
G12V	LVVVGAVGVGK	499.3142	37.1	y7	587.3511	1.00
				y9	785.4880	1.00
				y8	686.4196	0.73
				y4	360.2242	0.38
				b3	312.2282	0.32
G12D	LVVVGADGVGK	507.2935	18.0	y7	603.3097	1.00
				y9	801.4465	0.83
				y8	702.3781	0.66
				b3	312.2282	0.44
				y4	360.2242	0.36
G13D	LVVVGAGDVGK	507.2935	18.0	y7	603.3097	1.00
				y8	702.3781	0.62
				y9	801.4465	0.59
				y3	303.2027	0.42
				b3	312.2282	0.36

[a] iRT is a normalized retention time based on the 11 iRT peptides spiked into the sample

Not surprisingly, these two peptides co-eluted during the LC separation (see iRTs in Table 2). However, in their MS2 spectra, both peptides exhibited a diagnostic product ion that clearly distinguishes one from the other (Fig. 1). For the G12D peptide, the discriminating y4 ion was generated in high abundance; for the G13D peptide, while the discriminating y4 ion was not generated sufficiently, the y3 ion peak was much higher than that of G12D. This is likely due to the presence of aspartic acid at the N-terminal sides of D-G (for G12D) and D-V (for G13D) bonds which promotes amide bond cleavage during the collision-induced dissociation [33,34]. In samples containing low amounts of KRAS protein, G12D and G13D may be undistinguishable due to lack of y4 or y3 ions. In building the spectral library, LC-MS/MS analysis was performed separately to prevent interference by the mixing of product ions.

After building the spectral library, the targeted DIA assay was designed to cover the theoretical *m/z* values of all possible peptides harboring activating mutations in KRAS exon 2 (Table 1; shown non-redundant-amino acid changes). The isolation window for DIA in the selected mass range, 470–560, was set to 2 Th to reach the optimum efficiency of precursor transmission that reduces interference while maintaining high sensitivity. The DIA method was tested on a cell lysate mixed with five synthetic peptides. All five peptides were successfully identified with their corresponding mutation statuses (data not shown). Prior to application in tumor biopsy samples, the method was tested in a formalin-fixed colorectal cancer cell line (SW620) harboring G12V homozygous mutation. G12V mutation was successfully detected (Supplementary data, Fig. 1S).

3.2. Application in FFPE tumor samples with known RAS mutation status

Following verification in cell lines, the DIA assay was applied to 13 tumor biopsies from patients with cancers of the colorectum (n = 8), pancreas (n = 2), ampullary (n = 1), ovary (n = 1) and endometrium (n = 1) (Table 3). The RAS mutation status of each sample had been previously determined by DNA sequencing: 10 samples harbored KRAS mutations (G12A, G12D, G12V, and G13D) and 3 samples harbored NRAS mutations (G12D and G12V).

Targeted DIA analysis detected RAS wild-type peptide in all 13 tumor samples and RAS mutant peptides in 11 tumor samples (Fig. 2) (Supplementary data, Fig. 1S). The RAS mutation status by DIA in these 11 samples was concordant with the status by DNA sequencing (Table 3). Targeted DIA failed to detect mutant peptides in 2 samples (#9 and #10) likely due to the low intensities. Based on DNA analysis, both samples harbor G12V mutations. In other samples with known G12V mutations, G12V peptide exhibited poor chromatographic behavior (Fig. 2C and Fig. 1S). Its poor performance was confirmed in G12V-mutated SW620 cells (Supplementary data, Fig. 1S). Further optimization of the LC conditions may help to improve the assay for G12V

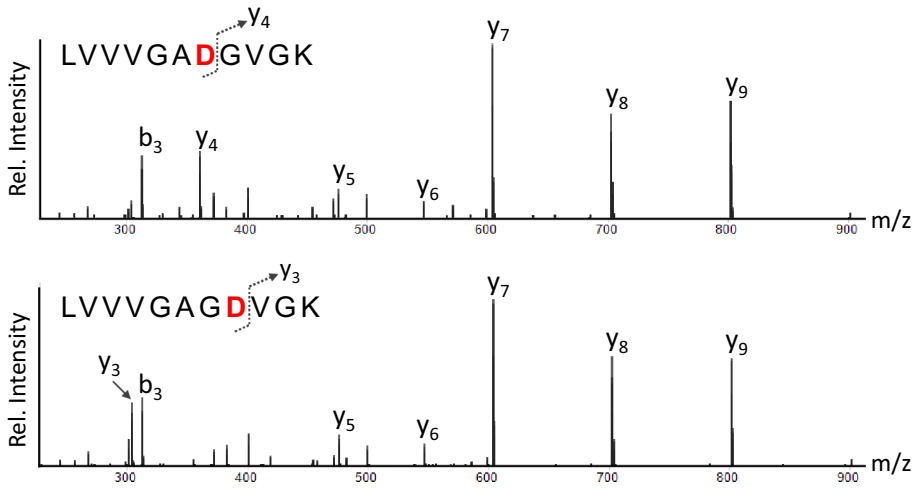

Fig. 1. MS2 spectra of LVVVGADGVGK (G12D) and LVVVGAGDVGK (G13D). Diagnostic product ion y4 was detected only in G12D peptide, and y3 was detected only in G13D peptide.

Table 3
RAS mutation testing by DNA sequencing and by DIA-based proteomics in 13 tumor biopsy samples.

Sample information		DNA Analysis			DIA-based Protein Analysis		
#	Indication	Gene	Mutation	RNA %	WT (AUC)	Mutant (AUC)	Ratio[a]
1	Colon Cancer	KRAS	G12A	65	29723	105851	0.78
2	Colon Cancer	KRAS	G12A	27	57052	52923	0.48
3	Colon Cancer	KRAS	G12D	31	34858	102815	0.75
4	Ovarian Cancer	KRAS	G12D	20	59105	91125	0.61
5	Colon Cancer	NRAS	G12D	35	34210	59008	0.63
6	Colon Cancer	KRAS	G12D	25	46637	84396	0.64
7	Colon Cancer	NRAS	G12D	21	64326	113717	0.64
8	Colon Cancer	KRAS	G12V	41	31640	4713	0.13
9	Pancreatic Cancer	KRAS	G12V	29	55032	NA	NA
10	Rectal Cancer	NRAS	G12V	26	42142	NA	NA
11	Pancreatic Cancer	KRAS	G12V	72	38181	6027	0.14
12	Ampullary Cancer	KRAS	G13D	38	25876	223660	0.90
13	Endometrial Cancer	KRAS	G13D	28	36712	109719	0.75

[a] Ratio = mutant peptide/[mutant peptide + WT peptide]

peptide by narrowing the peak width at the chromatography.

Samples with G12D and G13D mutations were clearly identifiable due to the presence of their diagnostic product ions. Even if the assay could not distinguish between the isobaric mutations of RAS proteins (G12V vs G13V), there is no clinically actionable difference between exon 2 mutations in codons 12 versus 13; a point mutation affecting either codon is predictive of therapeutic resistance. Therefore, the assay results are clinically informative.

3.3. Correlation of mutation yields between mRNA and protein

The DIA readouts of detected mutant peptides represent relative amounts of mutant RAS proteins in each sample. We hypothesized that the DIA readouts of mutant peptide and wild type peptide may provide a quantitative measure of mutation yields of RAS proteins. We used the ratios of DIA readouts (mutant peptide/[mutant + wild type peptide]) and compared this with the mutation yield at the mRNA level, measured as mutant reads/total reads (mRNA%, Table 3). Given that the ratio of mass spectrometric response factors between peptides ([mass spectrometric response factor for mutant peptide]/[mass spectrometric response factor for wild type peptide]) is constant for each mutant peptide, yields were compared within the same mutation type. Analysis for isobaric mutations such as G12D and G13D can be combined due to the nearly identical physicochemical properties of the corresponding peptides. For this comparison of mutation yield, we selected the tumor samples with G12D and G13D mutations (Fig. 3). A linear correlation ($r^2 = 0.51$) was observed between protein and mRNA analyses in these 7 tumor samples. Removal of one outlier (sample #5) yielded a strong correlation ($r^2 = 0.95$). Note that the peptide sequence LVVVGAGGVGK is common to all three RAS proteins (KRAS, NRAS, and HRAS), therefore DIA readouts from this part of the sequence cannot be deciphered into specific sub-types. However, ratios of mutant-to-total peptide would represent the presence of mutant RAS protein as compared to the normally functioning protein form regardless of sub-type. While there is no immediate clinical application of the mutation yields at the protein level, these results warrant further investigation with regard to potential relationships between quantities of RAS-mutant protein expression and therapeutic response.

3.4. Application in biopsy samples with failed DNA testing results

Following evaluation in samples with a known RAS mutation status, the assay was applied to two CRC biopsy samples with failed DNA testing results. The DIA-RAS assay identified one as wild type and the other as G13D mutated with the mutation yield of 0.52 (samples #14 and #15 in Supplementary information, Fig. 2S). The suggestion of wild type for sample #14 was based on the lack of detection of any mutant peptide within our library. Therefore, further library construction will help to draw more definitive conclusions. Note that DIA allows retrospective data analysis after updating the library to be used. The quantitative studies with dilution curves for all peptides are required to define the limit of quantification (LOQ) in order to develop this assay for routine clinical use.

4. Conclusions

A targeted, semi-quantitative DIA assay for detecting KRAS exon 2 mutations in codons 12 and 13 was developed and tested in FFPE tumor samples harboring known KRAS and NRAS mutations. From 13 RAS-mutated samples, the mutation statuses of 11 were successfully identified. The assay was then applied to two samples with failed DNA testing results and identified an additional G13D mutation. This proteomic method does not require prior knowledge of DNA status nor

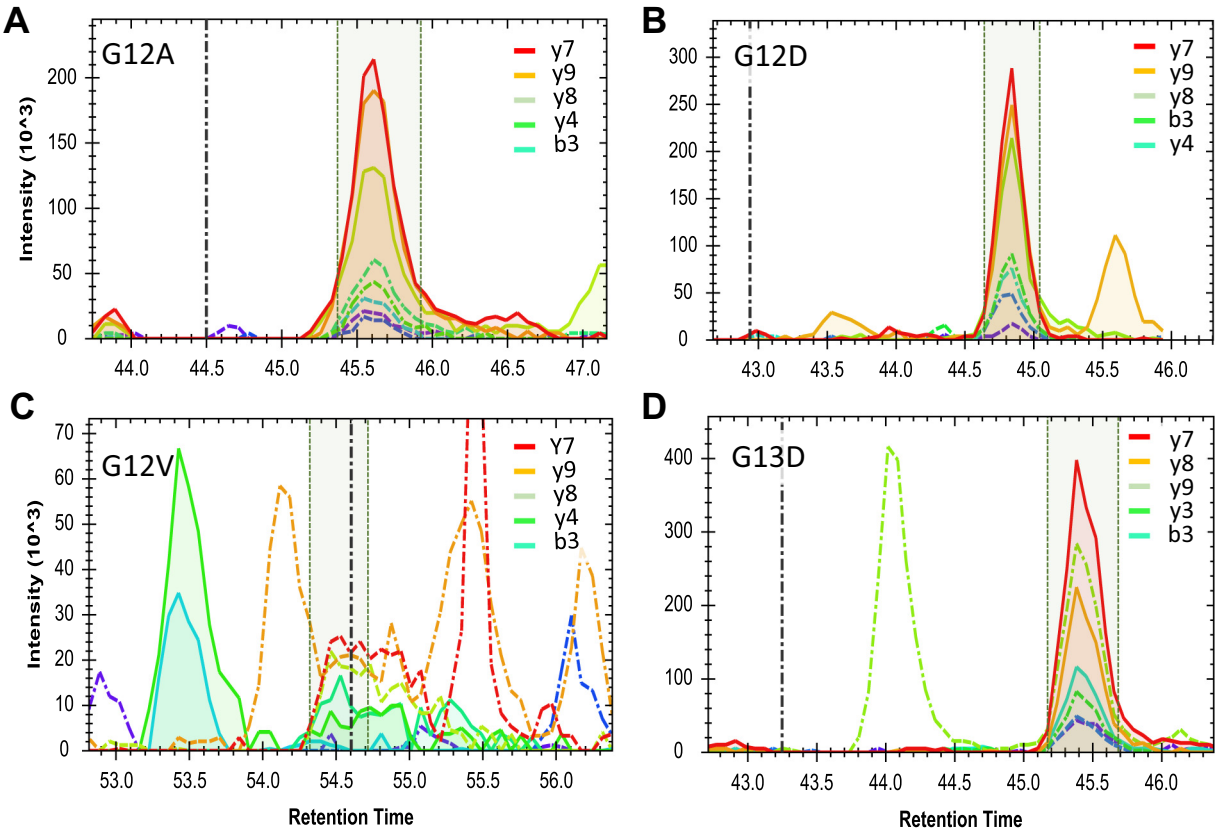

Fig. 2. KRAS exon 2 mutation analysis by targeted DIA in selected tumor biopsy samples. Chromatograms of product ions generated from (A) G12A, (B) G12D, (D) G12V, and (D) G13D peptides are shown. Shaded areas indicate the retention times used for quantitative analysis; potential interferences are indicated by broken lines. Vertical lines indicate the retention times of each peptide in the spectral library.

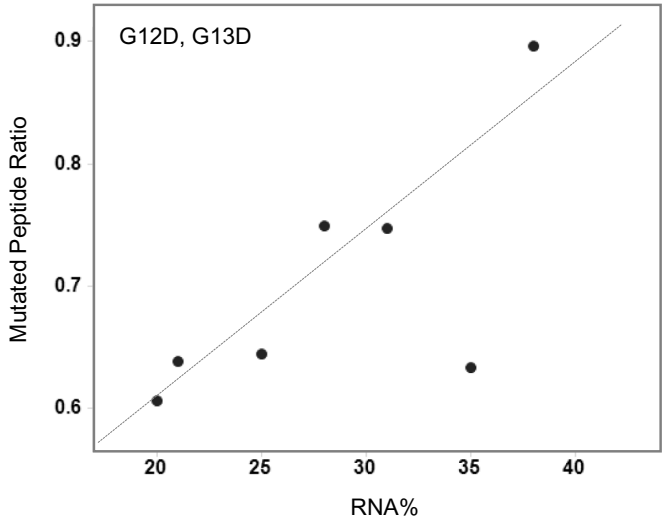

Fig. 3. Yields of mutant RAS peptide (mutant peptide/[mutant peptide + wild type peptide]) compared to yields of RAS-mutant RNA (mutant reads/total reads) in tumor biopsies with G12D and G13D mutations (n = 7).

enrichment for RAS proteins. Heavy SIL peptides are not necessary, yet building a high-quality spectral library for each peptide is crucial. The choice of *m/z* range for a gas-phase fractionation is flexible within a core range (478–528) given a 2 Th DIA isolation window throughout. G12V peptide exhibited poorer chromatographic performance than the other peptides used, requiring further efforts to optimize LC conditions.

Mutation yields were calculated using the normalized DIA readouts of wild type and mutated peptides, and demonstrated a linear correlation with yields of mutant mRNA. Although the assay as designed could not differentiate between mutations in different RAS oncogenes (KRAS, NRAS), additional proteomic analysis may enable this. In any case, such differentiation is not clinically critical as the existence of activating mutations in any RAS molecule is indicative of constitutive RAS signaling.

This robust method can inform therapeutic decision-making by providing solid evidence of mutant RAS proteins, and is applicable to tumor biopsy samples with insufficient sequenceable DNA. The biological and clinical significance of varying quantities of RAS mutant peptide warrants investigation to explore potential relationships between tumor expression levels of mutant RAS protein and response to therapy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2018.04.022.

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