Chromosomal translocations encoding chimeric fusion proteins constitute one of the most common mechanisms underlying oncogenic transformation in human cancer. Fusion peptides resulting from such oncogenic chimeric fusions, though unique to specific cancer subtypes, are unexplored as cancer biomarkers. Here we show, using an approach termed fusion peptide multiple reaction monitoring mass spectrometry, the direct identification of different cancer-specific fusion peptides arising from protein chimeras that are generated from the juxtaposition of heterologous genes fused by recurrent chromosomal translocations. Using fusion peptide multiple reaction monitoring mass spectrometry in a clinically relevant scenario, we demonstrate the specific, sensitive, and unambiguous detection of a specific diagnostic fusion peptide in clinical samples of anaplastic large cell lymphoma, but not in a diverse array of benign lymph nodes or other forms of primary malignant lymphomas and cancer-derived cell lines. Our studies highlight the utility of fusion peptides as cancer biomarkers and carry broad implications for the use of protein biomarkers in cancer detection and monitoring. Molecular & Cellular Proteomics 12: 10.1074/mcp. M113.029926, 2714–2723, 2013.

A cancer biomarker is generally an analyte that indicates the presence or extent of a specific form of cancer. A useful cancer biomarker should reliably distinguish between benign and malignant states and, ideally, distinguish one form of cancer from other, related differential diagnoses. Many human cancers contain recurrent chromosomal translocations and chimeric gene fusions that could be exploited as cancerspecific biomarkers (1, 2). Indeed, several structural aberrations are specific and pathognomonic for distinct types of cancer (3). Moreover, as new molecular therapies increasingly target oncogenic fusion proteins, the detection and quantitation of these proteins may also provide important, direct therapeutic guidance (4–6). Although genomic techniques targeting fusion partner genes are routinely used for diagnosing cancers, fusion peptides resulting from oncogenic chimeric fusions are unexplored as biomarker candidates for cancer detection. The specificity and qualitative/binary nature (i.e. present or absent) of fusion proteins in specific tumor types make these analytes attractive candidates for cancer detection.

Advances in mass spectrometry permit the direct and unbiased interrogation of proteins and peptides in complex mixtures with unambiguous identification of specific proteins (7, 8). Multiple reaction monitoring (MRM) via mass spectrometry is a powerful approach for the targeted detection of biomarker candidates in a complex background (9). MRM involves the focused interrogation of specific m/z windows for the precursor analyte, as well as selected fragment ions, following MS/MS analysis. By focusing only on specific m/z windows, one increases the sensitivity of detection dramatically, and within the context of a complex mixture there is the potential for a reproducible dynamic range spanning >4 orders of magnitude (10, 11).

Despite their enormous potential as biomarkers, fusion peptides resulting from oncogenic chimeric fusions have not been exploited for the specific and sensitive detection of...
cancer. Here we demonstrate the detection of unique fusion peptides that are specific for various forms of cancer. To demonstrate applicability in a clinically relevant scenario, we show the utility of our MRM-based MS approach combined with an innovative double stable isotope strategy for the identification of nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) fusion peptide arising from the corresponding chimeric fusion protein for the identification of NPM-ALK-positive anaplastic large cell lymphoma (ALCL). We show the exquisite specificity and sensitivity of this fusion peptide (FP) MRM approach and the extraordinary accuracy of its application with clinical biopsy material.

EXPERIMENTAL PROCEDURES

Materials—Peptides were purchased from Thermo Scientific. We procured the NPM-ALK fusion peptide (CGSGPVHISGQHLVYR) in three isotopically labeled forms: light (i.e. endogenous peptide); heavy, in which C-terminal arginine was 13C6/15N4 labeled; and double heavy, in which C-terminal arginine 13C6/15N4 and an internal valine 13C5/15N labeled. The N-terminal cysteine was carboxy-iodomethylated for all three. The concentration was determined by means of amino acid analysis, and the purity was >97%. Two API2-MALT1 fusion peptides were synthesized: ESRSVDGVSES and SRSVDGSVE (AQUA, purity >97%). BCR-ABL1 fusion peptides TINKEEL and HGDAEAL were also synthesized (purity >95%) along with the heavy isotopically labeled forms in which the C-terminal leucine was 13C6/15N labeled (AQUA, purity >97%). All other reagents were of the highest grade available.

Cell Lines—A total of nine cell lines derived from human T-cell lymphoproliferative disorders were cultured in RPMI supplemented with 10% fetal bovine serum, 2.05 mM L-glutamine, 1,000 UI/ml penicillin, 1,000 μg/ml streptomycin, and 2.5 μg/ml amphotericin B. We investigated five cell lines derived from ALK-positive ALCL and characterized by the chromosomal translocation t(2;5)(p23;q35); Karpass-299, SUP-M2, SR-786, SUD-HL-1, and DEL. We also studied three cell lines derived from ALK-negative cutaneous T-cell lymphoma: Mac-1, Mac-2A, and HH. Additionally, we analyzed the cell line Jurkat, which is representative of T-cell lymphoblastic leukemia.

Clinical Samples—This validation study was approved by the University of Michigan Institutional Review Board (RB # HUM00023256). In all, 23 clinical samples obtained through an internal biobank as cryopreserved cells were included in this study. All samples were collected at the time of diagnosis before any treatment. Cells were stored in liquid nitrogen until further analysis. After three washes with cold PBS to remove cryo-preservation solution, samples were processed in the same manner as the cell lines.

Detection and Quantitation of NPM-ALK via Quantitative Real-time Polymerase Chain Reaction—Total RNA was extracted by TRIzol (Invitrogen) and subjected to reverse transcription using SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. Quantitative real-time polymerase chain reaction was performed with the Mastercycler® ep realplex thermal cycler (Eppendorf, Hauppauge, NY). The expression levels of NPM-ALK RNA were determined using a TaqMan probe-based assay (forward primer: 5’-CAG-TGC-ATA-TTA-GTG-GAC-AGC-ACT-TAG-3’; reverse primer: 5’-TGA-TGG-TAG-AGG-TGA-GGA-3’; probe: 6-FAM-CAC-CAG-GAG-CTG-CAA-GCC-ATG-CA-TAMRA). Data analysis was done via the comparative Ct method with plasmid standard curves. Results were normalized to ABL (forward primer: 5’-CAG-CAC-CAC-ATG-TGG-GGA-CGA-3’; reverse primer: 5’-CTG-GAT-AAT-GGA-GCG-TGG-TG-3’; probe: 6-FAM-CAC-CCT-GGC-CGA-GTT-GST-TCA-T-TAMRA).

Detection and Quantitation of NPM-ALK via Western Blot—Four micrograms of protein were resolved on 10% SDS-PAGE gels, transferred onto nitrocellulose membrane, probed with individual antibodies, and visualized using an enhanced chemiluminescence detection system. The primary antibodies were anti-ALK antibody (Invitrogen) and anti-β-actin (Sigma). The relative expression of NPM-ALK was determined using ImageJ software.

Protein Extraction and Digestion—Cells were lysed in 1 ml of lysis buffer containing 8 M urea/0.4 M ammonium bicarbonate/0.1% SDS with sonication on ice and then spun at 16,000g for 10 min. For each sample, 0.5 mg of protein were reduced with 10 mM Tris (2-carboxyethyl) phosphate for 60 min at 60 °C and then alkylated with 12 mM iodoacetamide for 30 min at room temperature in the dark. Samples were diluted 4-fold with 100 mM potassium phosphate pH 8.0 and then digested with trypsin overnight at 37 °C using an enzyme-to-protein ratio of 1/50 (w/w). After acidification with 6N HCl, samples were desalted on a C18 cartridge (Sep-Pak plus C18 cartridge, Waters, Milford, MA). Purified peptides were eluted with 30% acetonitrile/0.1% trifluoroacetic acid and dried before any further processing. Each sample was prepared in triplicate.

High-performance Liquid Chromatography—Dried peptides (0.5 mg) from either cell lysates derived from cell lines or primary tumor samples were dissolved in 250 μl 1% acetic acid and 2% acetonitrile to create a 2μg/μl peptide solution. A standard curve was prepared by spiking heavy NPM-ALK peptide C(57.0215)GSGPVHISGQHLVYR(13C6/15N4) into a portion of the peptide solution and then serially diluting with unspiked peptide solution (0.439 to 113 femole/μl). Another portion of the peptide solution was spiked with double heavy NPM-ALK peptide C(57.0215)GSGPVHISGQHLVYR(13C6/15N4) to serve as an internal standard. This was added to each standard level prior to injection for LC-MS/MS (43.9 femole/μl). Peptide samples were separated using an in-house packed reversed-phase column (Magic C18 AQ, 200 Å, 5 μ (Michrom Bioreources, Inc., Auburn, CA), 150 μm inner diameter × 100 mm) on an LC Paradigm MS4 system (Michrom Bioreources, Inc.). Samples were injected using a Paradigm AS1 autosampler (Michrom Bioreources, Inc.). After 2 μl of each peptide sample was loaded onto the column, the LC gradient was initiated at a 1.1 μl/min flow rate with 92% mobile phase A (1% acetic acid and 2% acetonitril) and 8% mobile phase B (1% acetic acid and 96% acetonitril). The flow rate was increased to 1.3 μl/min and mobile phase B was increased to 43% over the next 60 min. Mobile phase B was brought to 99% over the next 5 min and held there for an additional 5 min. The flow rate and mobile phase composition were then brought back to the initial conditions over 5 min and held there for an additional 15 min to equilibrate the column.

Fusion Peptide Multiple Reaction Monitoring Mass Spectrometry—A TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Scientific) with a Silver-TSQ Ultra-Exactive MS source (Michrom Bioresearches, Inc.) was used for all experiments. A number of FPs were screened via MRM-MS. To detect the API2-MALT1 (ex7/ex5), BCR-ABL1 (e13a2), and BCR-ABL1 (e1a2) FPs, we utilized the parameters described in supplemental Table S1. Chromatography conditions and linearity were also established for each (supplemental Fig. S1). Given our laboratories long-standing interest in ALCL and access to ALK-positive ALCL clinical samples, we selected the NPM-ALK fusion peptide for further analysis to establish the feasibility and robustness of FP-MRM-MS for preclinical validation. MS parameters for the NPM-ALK FP were as follows: spray voltage, 1600 V; capillary temperature, 220 °C; skimmer offset, −5. Transitions and parameters for each NPM-ALK FP (Table I) were initially selected using Pinpoint Quantitation Software (version 1.1.7) (Thermo Fisher Scientific).

The NPM-ALK fusion peptide (CGSGPVHISGQHLVYR) generated via digestion with trypsin contains an NH2-terminal cysteine. Carbamidomethylation of NH2-terminal cysteine can cause peptides to
undergo spontaneous cyclization in a pH- and time-dependent manner, resulting in a cyclized and an uncyclized form of the peptide (supplemental Fig. S2) (12). LC-MS/MS analysis of the NPM-ALK FP indicated the presence of cyclized and uncyclized forms with different hydrophobicity characteristics, resulting in separate elution times in a standard reverse-phase gradient system (supplemental Fig. S2A). We infused the three peptides individually into a triple quadrupole mass spectrometer and observed +2 and +3 charge states, with the +3 charge state being the most abundant ion for both cyclized and uncyclized forms (supplemental Fig. S2B). Both the cyclized and the uncyclized +2 and +3 charged precursor ions were selected for monitoring, along with five to six product ions for each isotopic variant. Only y and b ion series product ions with m/z greater than the precursor m/z were chosen (13). The optimized parameters in terms of collision energy and product ion selection for Q3 transition are presented in Table I. Results presented are based upon the cyclized +3 form of the NPM-ALK FP that provided the greatest signal intensity. Although we monitored several Q3 transitions for unambiguous identification of the target peptide, we chose the y5 ion for quantification, as it was the most intense ion that exhibited a higher mass than the precursor peptide (supplemental Figs. S3 and S4). Representative extracted ion chromatograms are shown for the cyclized +3 charged NPM-ALK peptide (Fig. 1C and supplemental Fig. S4).

MRM was carried out in positive ion mode with the following conditions: scan width, 0.002 m/z; scan time, 0.020 s; quadrupole 1, 0.70 full width at half-maximum; quadrupole 3, 0.70 full width at half-maximum; collision gas pressure, 1.5 mTorr; chromatography filter peak width, 5 s. Data were collected in centroid mode and processed using Xcalibur (version 2.0.6) (Thermo Electron Corporation, Waltham, MA). Processed data were then exported to Excel 2010 (Microsoft, Redmond, WA), and the endogenous NPM-ALK peptide values were calculated by inserting the light NPM-ALK peptide area ratios into the linear equation obtained from the heavy/double heavy NPM-ALK peptide curve.
The limit of detection (LOD) and limit of quantitation (LOQ) were determined for each curve. The LOD was determined by the lowest standard that gave a signal/noise ratio of \( \text{H}_{10} \) and its calculated value based upon the standard curve was 20% different than the theoretical value.

**RESULTS**

**Identification of Fusion Peptides from Chimeric Fusions and Selection of a Model System**—To determine whether chimeric fusion proteins arising from chromosomal translocations yield specific FPs upon digestion with common proteases, we evaluated 26 chimeric fusions known to occur in a spectrum of human cancers. We had two criteria for qualification of a peptide as suitable for FP-MRM analysis: (i) amino acids from both fusion partners contribute to the composition of the FP, and (ii) commonly used proteolytic enzymes generate a FP that is suitable for MS analysis. As indicated in Table II, all of the 26 chimeric fusions that we examined generate FPs that meet our criteria. We established chromatography, linearity, and MRM parameters for FPs derived from NPM-ALK, API2-MALT1, and BCR-ABL1 that are diagnostic of NPM-ALK-positive ALCL, extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue, and chronic myeloid leukemia, respectively (See "Experimental Procedures," Figs. 1–E, supplemental Figs. S1–S4, Table I, and supplemental Table S1). We then selected NPM-ALK-positive ALCL as the model system to demonstrate the utility of FPs as biomarkers for specific cancer detection in a clinically relevant scenario.

The limit of detection (LOD) and limit of quantitation (LOQ) were determined for each curve. The LOD was determined by the lowest standard that gave a signal/noise ratio of \( \text{H}_{10} \) and its calculated value based upon the standard curve was 20% different than the theoretical value.

**Development of a Double Stable Isotope Labeling Strategy for Quantitative Assessment of Endogenous NPM-ALK Fusion Peptide via LC-MRM**—The presence of other peptides and small molecules in the matrix can suppress or enhance the signal of the target analyte (11). Pooled normal serum, body fluids, or cell lines that do not express the target analyte are background matrices commonly used to generate a standard curve using LC-MRM against which the test sample response

---

**Table I**

| NPM-ALK peptide sequence | Charge state | Light Charge | Heavy Charge | Double heavy Charge | Collision energy | Fragment ion |
|--------------------------|-------------|-------------|--------------|--------------------|------------------|-------------|
|                          |             | Q1         | Q3           | Q1          | Q3          |             |
| C(C57.021464)GSGPVHISGQLVYR | +2          | 934.476    | 1058.574    | 938.480    | 1068.574    | 941.476     | 1074.574    | 38         | \( y_9 \)  |
|                          |             | 1080.489   | 1080.489    | 1087.542   | 1087.542    | 1087.542    | \( y_9 \)  |
|                          |             | 1171.658   | 1181.658    | 1232.717   | 1232.717    | 1232.717    | \( y_9 \)  |
|                          |             | 1308.717   | 1318.717    | 1330.632   | 1330.632    | 1330.632    | \( y_9 \)  |
|                          |             | 1330.632   | 1330.632    | 1330.632   | 1330.632    | 1330.632    | \( y_9 \)  |
| C(C57.021464)GSGPVHISGQLVYR | +3          | 622.890    | 649.403     | 626.100    | 659.403     | 627.966     | 655.403     | 30         | \( y_5 \)  |
|                          |             | 768.462    | 796.462     | 796.462    | 802.462     | 796.462     | 802.462     | \( y_5 \)  |
|                          |             | 971.542    | 981.542     | 987.542    | 987.542     | 987.542     | \( y_5 \)  |
|                          |             | 1058.574   | 1068.574    | 1074.574   | 1074.574    | 1074.574    | \( y_5 \)  |
|                          |             | 1080.489   | 1080.489    | 1080.489   | 1080.489    | 1080.489    | \( y_5 \)  |
|                          |             | 1407.786   | 1417.786    | 1423.786   | 1423.786    | 1423.786    | \( y_5 \)  |
| C(C39.994915)GSGPVHISGQLVYR Cyclized | +2          | 924.962    | 791.351     | 1171.658   | 932.962     | 791.351     | 932.962     | 38         | \( b_8 \)  |
|                          |             | 971.542    | 981.542     | 1187.658   | 987.542     | 987.542     | \( b_8 \)  |
|                          |             | 1058.574   | 1068.574    | 1187.658   | 1074.574    | 1074.574    | \( b_8 \)  |
|                          |             | 1171.658   | 1181.658    | 1324.717   | 1187.658    | 1187.658    | \( b_8 \)  |
|                          |             | 1308.717   | 1318.717    | 1373.717   | 1373.717    | 1373.717    | \( b_8 \)  |
| C(C39.994915)GSGPVHISGQLVYR Cyclized | +3          | 617.31     | 649.403     | 620.410    | 659.403     | 622.310     | 655.403     | 30         | \( b_9 \)  |
|                          |             | 678.266    | 791.351     | 791.351    | 791.351     | 791.351     | \( b_9 \)  |
|                          |             | 971.542    | 981.542     | 987.542    | 987.542     | 987.542     | \( b_9 \)  |
|                          |             | 1058.574   | 1068.574    | 1074.574   | 1074.574    | 1074.574    | \( b_9 \)  |
|                          |             | 1171.658   | 1181.658    | 1187.658   | 1187.658    | 1187.658    | \( b_9 \)  |
|                          |             | 1308.717   | 1318.717    | 1373.717   | 1373.717    | 1373.717    | \( b_9 \)  |

*“Heavy” and “double heavy” refer to the isotopic versions of the peptide.*

*a* Represents the most intense fragment ion.

The heavy peptides contain an isotopically labeled arginine \( (13C_6/15N_4) \) at the C-terminus making them 10 Da heavier than their light counterparts.

The double heavy peptides contain both an isotopically labeled arginine \( (13C_6/15N_4) \) at the C-terminus and an isotopically labeled valine \( (13C_5/15N) \) making them 16 Da heavier than their light counterparts.

Red: amino acid residues contributed by fusion partner 1.

Blue: amino acid residues contributed by fusion partner 2.

The limit of detection (LOD) and limit of quantitation (LOQ) were determined for each curve. The LOD was determined by the lowest standard that gave a signal/noise ratio of \( >10 \). The LOQ was determined by the lowest standard that gave a signal/noise ratio of \( >10 \), and its calculated value based upon the standard curve was \( \pm 20 \% \) different than the theoretical value.
TABLE II
Examples of chimeric fusion proteins and their translocations from several cancer types showing the predicted fusion peptides that are amenable to FP-MRM-MS using the proteases indicated

| Cytogenetic abnormality | Fusion protein | Disease | Fusion peptide | Protease |
|------------------------|----------------|---------|----------------|---------|
| Hematological neoplasm (leukemia/lymphoma) | NPM-ALK* | ALK-positive ALCL | CGSGBP/HISQGHLVYYR | Trypsin |
| t(2;5)(p23;q35) | BCR-ABL1 (E13a2)* | CML, B-ALL | TINKEAL | Chymotrypsin |
| t(2;5)(p23;q35) | BCR-ABL1 (E12a2) | CML, B-ALL | HGDAEL | Chymotrypsin |
| t(11;18)(p11;q21) | API2-MALT1 ( exon-5) | MALT lymphoma | SRSYG/GE | V8-DE |
| t(11;18)(p11;q21) | API2-MALT1 ( exon-6) | MALT lymphoma | ESRSVVG/SEK | Lyn-C |
| t(2;9)(p23;q21) | TPM3-ALK | ALK-positive ALCL | TIDDELYR | Trypsin |
| t(11;16)(p11;q21) | CLTC-ALK | ALK-positive DBLCL and ALCL | LPGHV/ADHP/PPA/YYR | Trypsin |
| t(2;5)(p23;q35) | SQSTM1-ALK | ALK-positive DBLCL | NVGESVAALSPL/YYR | Trypsin |
| t(4;11)(q21;23) | MLL-AF4 | Biphenotypic ALL, B-ALL | FKOTYSNE | V8-DE |
| t(9;11)(p22;q33) | MLL-AF9 | AML | SDFVYCVCCEP/HHK | Trypsin |
| t(8;21)(q22;q22) | AMI1-MTG8 | AML | ITYDG/PRPRN/RTK | Lyn-C |
| t(11;19)(p13;q15) | NUP98-HOXD13 | AML | GAPQAPGD/VAL | Chymotrypsin |
| t(11;16)(p11;q21) | MLL-ELL | AML | VDKFD/SL/RL | Arg-C |
| t(5;17)(q22;p11) | PML-RARA | APL | LSSSOTQG/KAE | V8-DE |
| inv(2)(p21;q22) | EML4-ALK (Variant 1) | NSCLC | PTPKPGPKP/RRKHOE | V8-DE |
| inv(2)(p21;q22) | EML4-ALK (Variant 2) | NSCLC | YIMNSN/DSYEL/LYR | Trypsin |
| inv(2)(p21;q22) | EML4-ALK (Variant 3a) | NSCLC | KNSOVRK/HOE | V8-DE |
| inv(7)(q21;q34) | AKAP9-BRAF | Papillary thyroid carcinoma | SEQDLIR | Trypsin |
| t(18;18)(p11.2;q11.2) | SYT-SSX1 | Synovial sarcoma | QIMPKP/PAE | V8-DE |
| t(18;18)(p11.2;q11.2) | SYT-SSX2 | Synovial sarcoma | QIMPKP/PAE | V8-DE |
| t(2;22)(p21;q11) | EWSR1-ERG | Ewing sarcoma | GQGQS/QQ/OL | Chymotrypsin |
| t(2;22)(p21;q11) | EWSR1-ERG | Ewing sarcoma | GQGQS/QQ/OL | Chymotrypsin |
| t(1;22)(q13;p12) | EWS/WT1 | DSRCT | GGQDS/EXPY | Chymotrypsin |
| t(1;22)(q13;p12) | EWS/WT1 (fusion type 1) | Clear Cell sarcoma | GGQ/MKL/SSD/ETDR | Arg-C |
| t(1;22)(q13;p12) | EWSR1/ATF1 (fusion type 2) | Clear Cell sarcoma | GQQAIA/PNAL | Chymotrypsin |
| t(1;18)(p22;q13) | COL1A1-PDGFB | Dermatofibrosarcoma protuberans | QGPSGASGPAG/PRG | V8-DE |

* Detected via fusion peptide multiple reaction monitoring mass spectrometry in this study.
Red: amino acid residues contributed by fusion partner 1.
Blue: amino acid residues contributed by fusion partner 2.

ALK-positive ALCL, anaplastic large cell lymphoma; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; B-ALL, B-cell acute lymphoblastic leukemia; CML, chronic myeloid leukemia; DBLCL, diffuse large B-cell lymphoma; DSRCT, desmoplastic small round cell tumor; MALT, mucosa-associated lymphoid tissue; NSCLC, non-small cell lung carcinoma.

is compared. However, these are still prone to matrix-associated errors in quantitation (11). In order to ensure that our assay accurately measured endogenous NPM-ALK levels in different matrices, we devised an innovative quantitation strategy using two stable-isotope-labeled NPM-ALK peptides with LC-MS and FP-MRM (see "Experimental Procedures"). In this assay, heavy and double heavy NPM-ALK peptides were used to generate a standard curve directly in each test matrix allowing the endogenous NPM-ALK peptide response to be determined from the same injections (Figs. 1D and 1E). By establishing our standard curve directly in the sample matrix, we eliminated potential quantitation errors seen when the analyte responses of the standard curve matrix and the sample matrix differ (11).

Quantitation of NPM-ALK Fusion Peptide Expression via FP-MRM in Cell Lines—Once the conditions for FP-MRM had been established, a series of NPM-ALK-positive and -negative cell lysates were evaluated using the approach described (Fig. 1B). Protein lysates derived from five NPM-ALK-positive cell lines (SUD-HL-1, Del, Karpas-299, Sup-M2, and SR786) and four NPM-ALK-negative lymphoma cell lines (HH, Mac-1, Mac-2A, and Jurkat) were analyzed. A representative standard curve in an NPM-ALK-positive background (SR786) demonstrates linearity from 0.439 to 113 fmole/mg (220 to 56,500 fmole/mg lysate) (Fig. 1D). A representative extracted ion chromatogram of the ion used for quantitation revealing co-elution of both endogenous and exogenously added peptides is shown in Fig. 1C and supplemental Fig. S4A. Representative extracted ion chromatograms showing co-elution of all the ions monitored for the light, heavy, and double heavy +3 cyclized form of the NPM-ALK FP are shown in supplemental Figs. S4B–S4D. Using FP-MRM-MS, we successfully identified and quantitated endogenous NPM-ALK peptide in five NPM-ALK-positive cell lines with a coefficient of variation of <20% (Fig. 2A). None of the four NPM-ALK-negative cell lines exhibited detectable levels of the target analyte demonstrating absolute specificity (Fig. 2A). Across all cell lysates analyzed using this approach, the LOD ranged from 0.439 to 0.879 fmole/μl (220 to 440 fmole/mg lysate) and the LOQ ranged from 0.439 to 3.52 fmole/μl (220 to 1,760 fmole/mg lysate).
lysate) (Fig. 2A). The results of FP-MRM analysis across the nine cell lines were concordant with both quantitative real-time polymerase chain reaction (Fig. 2B) and Western blot analysis (Figs. 2C and 2D). Sensitivity and specificity for this method were calculated using cell lysate results that matched perfectly between experimental and expected. This resulted in a sensitivity of 5/5 = 100% and a specificity of 4/4 = 100%. The calculated standard concentrations across all nine cell lysates are shown in supplemental Table S2A. To assess the sensitivity at the cellular level at which FP-MRM analysis could detect the NPM-ALK FP, a series of dilutions were prepared using an NPM-ALK-positive cell (SUD-HL-1) and a negative cell (Mac1) (Fig. 3). The NPM-ALK FP was detectable above the limit of detection at 5% NPM-ALK-positive cells (SUD-HL-1) in a background of NPM-ALK-negative cells (Mac1) and above the limit of quantitation at 15% NPM-ALK-positive cells (SUD-HL-1) in a negative background (Mac1) (Fig. 3A). FP-MRM results for the dilutions were concordant with both quantitative real-time polymerase chain reaction (Fig. 3B) and Western blot analysis (Figs. 3C and 3D).

Quantification of NPM-ALK Fusion Peptide Expression via FP-MRM in Patient Samples—To assess the utility of the NPM-ALK FP-MRM approach in routine clinical patient samples, we blindly interrogated a cohort of 23 patient samples via FP-MRM for NPM-ALK status on lysates obtained from benign hyperplasias, a variety of B- or T-cell lymphomas, and NPM-ALK-positive ALCL lymph node biopsy specimens (supplemental Table S3). A representative standard curve for NPM-ALK-positive patient sample 18 demonstrates linearity from 0.439 to 113 fmole/µL (220 to 56,500 fmole/mg lysate) (Fig. 1E). Across patient samples, the LOD ranged from 0.439 to 1.76 fmole/µL (220 to 880 fmole/mg lysate), and the LOQ ranged from 0.439 to 3.52 fmole/µL (220 to 1,760 fmole/mg lysate) (Fig. 4A). FP-MRM analysis correctly determined the NPM-ALK peptide status in all 23 patient samples (Fig. 4A). Because of limited samples, 5 of the 23 patient samples were evaluated against a single 50-fmole/µL heavy and double heavy internal standard and analyzed in triplicate via LC-MRM. Eleven patient samples were NPM-ALK positive, and 12 were negative. These results were perfectly concordant with those obtained by means of Western blot analysis (Figs. 4B and 4C) and consistent with the original histopathologic diagnoses. Thus, the FP-MRM approach yielded a sensitivity of 11/11 = 100% and a specificity of 12/12 = 100%.

**Table 1.** Relative expression level of NPM-ALK fusion peptides normalized to 10,000 copies of ABL

| NPM-ALK FP-MRM approach          | LOD (fmole/µL) | LOQ (fmole/µL) | %CV | Technical Replicate |
|----------------------------------|----------------|----------------|-----|---------------------|
| NPM-ALK +                         | 0.439          | 0.439          | 9   | 9                   |
| Karpas-299                       | 1.76           | 0.439          | 15  | 9                   |
| SUP-M2                           | 0.879          | 0.439          | 15  | 9                   |
| Del                              | 0.439          | 0.439          | 9   | 9                   |
| SUD-HL-1                         | 0.439          | 0.439          | 9   | 9                   |
| HH                               | < LOD          | 0.439          | NA  | 9                   |
| Mac-1                            | < LOD          | 0.879          | NA  | 9                   |
| Mac-2A                           | < LOD          | 0.439          | NA  | 9                   |
| Jurkat                           | < LOD          | 0.439          | NA  | 9                   |

NA = Not applicable
LOQ = Lowest heavy NPM-ALK standard observed with S/N > 10 and % difference from theoretical ≤ 20%
LOD = Lowest heavy NPM-ALK standard observed with S/N > 10

**Fig. 2. Detection of NPM-ALK fusion peptide in cell lines.** Five NPM-ALK-positive and four NPM-ALK-negative cell lysates were analyzed using different techniques. A, cell lysate quantitation results obtained using our FP-MRM approach were in agreement with (B) those obtained via the quantitation of NPM-ALK expression by means of quantitative real-time PCR. mRNA was quantified using a TaqMan probe-based assay. The expression level of NPM-ALK transcript was normalized to the expression levels of ABL for each cell line. Data represent the mean of three biological replicates, and error bars represent S.D. C, NPM-ALK expression from Western blot analysis was also in agreement with the corresponding FP-MRM data. D, quantitation of the Western blot results for NPM-ALK expression is shown. The expression level of NPM-ALK was normalized to the expression levels of β-actin for each cell line. Data represent the mean of three biological replicates, and error bars represent S.D.
calculated standard concentrations across patient samples are shown in supplemental Table S2B.

**DISCUSSION**

We demonstrate herein the utilization of MRM-MS for the detection of fusion chimeras resulting from recurrent chromosomal translocations characteristic of specific forms of cancer. We detected five unique fusion peptides from five distinct chimeric fusion proteins that are characteristic of three different types of cancer (NPM-ALK in ALCL, API2-MALT1 in MALT lymphoma, and BCR-ABL in chronic myeloid leukemia). Using FP-MRM-MS, we performed a detailed analysis of the specific NPM-ALK fusion encoded by t(2;5)(p23;q35) for the detection of ALCL in a clinically relevant context. Using this approach, we correctly identified NPM-ALK-positive and -negative cell lines from a large cohort of clinical samples, including those carrying the NPM-ALK fusion (Figs. 2 and 4). NPM-ALK FP-MRM results show excellent qualitative correlation with orthogonal assays such as Western blotting and quantitative real-time polymerase chain reaction (Figs. 2–4). Dilutional studies revealed that FP-MRM exhibited sensitivity comparable to that of Western blotting, approximating 250 fmole/mg of tumor protein (Fig. 3). Using cellular dilution studies, we demonstrated the ability to detect NPM-ALK fusion peptides when the NPM-ALK-positive lymphoma cells constituted only 5% of the cellular composition of the interrogated protein extract (Fig. 3). As shown in this study, this level of sensitivity is well suited for the detection of tumor cells in clinical biopsy specimens with >5% tumor involvement. Importantly, FP-MRM using actual clinical samples of ALCL carrying the t(2;5)(p23;q35) aberration and expressing the NPM-ALK chimeric protein yielded 100% specificity with no false positives or negatives (Fig. 4).

The use of novel protein biomarkers has been limited by the availability of suitable techniques for their accurate detection (15). The high false-discovery rates of large-scale methods of analyte interrogation, the low levels of the candidate biomarkers, and the inherently variable signal-to-noise ratios of immunologic assays have remained significant barriers. The FP-MRM-based approach described herein for the diagnostic
**A**

| Patient ID | Endogenous NPM-ALK fmole/µL | FP-MRM Interpretation (Blind) | LOQ fmole/µL | LOD fmole/µL | %CV | Technical Replicate |
|------------|-----------------------------|-------------------------------|--------------|--------------|-----|---------------------|
| 1          | 3.08                        | +                             | 0.879        | 0.439        | 13  | 9                   |
| 2          | 5.88                        | +                             | 0.879        | 0.439        | 11  | 9                   |
| 3          | 5.79                        | +                             | 0.439        | 0.439        | 8   | 9                   |
| 4          | 2.56                        | +                             | 1.76         | 0.879        | 28  | 9                   |
| 5*         | ND                          | -                             | -            | -            | NA  | 3                   |
| 6          | < LOD                       | -                             | 0.439        | 0.439        | NA  | 9                   |
| 7          | < LOD                       | -                             | 1.76         | 1.76         | NA  | 9                   |
| 8          | < LOD                       | -                             | 0.879        | 0.879        | NA  | 9                   |
| 9*         | ND                          | -                             | -            | -            | NA  | 3                   |
| 10*        | ND                          | -                             | -            | -            | NA  | 3                   |
| 11*        | ND                          | -                             | -            | -            | NA  | 3                   |
| 12*        | ND                          | -                             | -            | -            | NA  | 3                   |
| 13         | < LOD                       | -                             | 0.439        | 0.439        | NA  | 9                   |
| 14         | < LOD                       | -                             | 0.879        | 0.879        | NA  | 9                   |
| 15         | < LOD                       | -                             | 0.879        | 0.439        | NA  | 8                   |
| 16         | < LOD                       | -                             | 3.52         | 0.439        | NA  | 9                   |
| 17         | 5.92                        | +                             | 1.76         | 0.439        | 9   | 9                   |
| 18         | 7.1                         | +                             | 0.879        | 0.439        | 13  | 9                   |
| 19         | 2.86                        | +                             | 0.879        | 0.439        | 18  | 9                   |
| 20         | 4.62                        | +                             | 0.879        | 0.439        | 14  | 9                   |
| 21         | 3.01                        | +                             | 0.879        | 0.439        | 22  | 9                   |
| 22         | 13.7                        | +                             | 1.76         | 1.76         | 11  | 9                   |
| 23         | 12.2                        | +                             | 1.76         | 0.879        | 18  | 9                   |

* = Patients evaluated using 50 fmole/µl heavy & double heavy NPM-ALK
ND = Not detected
NA = Not applicable
LOQ = Lowest heavy NPM-ALK standard observed with S/N > 10
and % difference from theoretical ≤ 20%
LOD = Lowest heavy NPM-ALK standard observed with S/N > 10

**B**

- **Patient 1**
- **Patient 2**
- **Patient 3**
- **Patient 4**
- **Patient 5**
- **Patient 6**
- **Patient 7**
- **Patient 8**
- **Patient 9**
- **Patient 10**
- **Patient 11**
- **Patient 12**
- **Patient 13**
- **Patient 14**
- **Patient 15**
- **Patient 16**
- **Patient 17**
- **Patient 18**
- **Patient 19**
- **Patient 20**
- **Patient 21**
- **Patient 22**
- **Patient 23**

**C**

- **Relative expression of NPM-ALK**

---

**Fig. 4.** FP-MRM quantitation of NPM-ALK fusion peptide from clinical patient samples. **A,** 23 patient samples were analyzed using FP-MRM to ascertain the presence of NPM-ALK fusion peptide. Clinical patient samples had previously been identified as being NPM-ALK-positive ALCLs (11) or negative (12) [supplemental Table S5]. Asterisks indicate patients evaluated against a single 50-fmole/µl heavy and double heavy internal standard and analyzed in triplicate. **B,** NPM-ALK expression determined via Western blot analysis. Four micrograms of protein were run for Western blot analysis to detect and quantify NPM-ALK. **C,** quantitation of the Western blot results for NPM-ALK expression. The expression level of NPM-ALK was normalized to the expression levels of β-actin for each patient sample.
Fusion Peptides for Cancer Detection

detection of chimeric fusion proteins offers several advantages that are apparent from the results of our study. Through the targeted monitoring of multiple transitions, the approach provides exquisite specificity, as observed in our study. This approach permitted high-sensitivity detection of NPM-ALK fusion at levels as low as 0.439 fmole/μl (220 fmoles/μl lysate) without the need for fusion protein-specific antibody enrichment or target amplification strategies (as are typically employed for immunophenotypic or polymerase chain reaction (PCR)-based detection of oncogenic chimeric fusions). Specifically in this regard, the lack of necessity for nucleic acid amplification is advantageous, as it obviates PCR-associated contamination issues (16). The implementation of accurate protein biomarkers in clinically useful scenarios has been challenging because many biomarker candidates are expressed in nondisease states, and thus the establishment of universal quantitative threshold levels for the unequivocal diagnosis of disease is difficult. In this regard, chimeric fusion proteins arising from chromosomal translocations are ideal biomarkers because of their qualitative nature and pathognomonic specificity in several forms of cancer. Overall, the approach is fairly simple to implement and scalable for the analysis of multiple samples and specimen types.

The incorporation of two mass-discriminable synthetic stable-isotope-labeled peptides into the assay design facilitates the accurate detection and quantification of endogenous NPM-ALK fusion peptides in any sample type. This simple “heavy/double heavy”-labeled internal standard strategy permits standard curve construction for every sample, and an area ratio (heavy/double heavy) may be calculated for each point on the calibration curve. This approach allows for the determination of the LOQ for each individual sample, providing greater confidence that the LOQ accurately reflects both the instrument performance at the time of analysis and matrix differences between samples that could affect quantification. With this approach, accurate determination of the levels of endogenous analyte are thus possible even at the LOD, as potential matrix effect differences between samples are essentially negated.

In conclusion, our study serves as proof of principle that FP-MRM-MS is an effective strategy for the detection of chimeric oncogenic fusion proteins. The approach is readily applicable for the diagnostic detection of fusion peptides arising from chimeric fusion proteins encoded by genes involved in chromosomal translocations. It is readily amenable to multiplexing such that multiple fusion peptides and their transitions may be monitored at femtomolar sensitivity (10, 11, 17). Given its advantages, we anticipate that MRM-based detection of fusion peptides will be exploited for the specific detection of cancers in clinically relevant contexts. In particular, this technology could be employed for quantitative monitoring of fusion proteins following targeted therapies.

Acknowledgements—We thank Dr. Damian Fermin for MS/MS data support and Dr. Thomas C. King for critically reviewing the manuscript.

Author contributions: K.P.C., V.B., and D.R. performed research. K.P.C., V.B., D.R., and T.W. analyzed data. K.P.C., V.B., D.R., A.I.N., M.J.M., M.S.L., and K.S.J.E.J. wrote the paper. M.S.L. and K.S.J.E.J. mentored the project.

* K.E.J. was supported by NIH Grant Nos. R01 DE119249 and R01 CA136905. M.S.L. was supported by NIH Grant No. R01 CA140806. [This article contains supplemental material.]

To whom correspondence should be addressed: Kojo S. J. El-enitoba-Johnson, MD, Henry Clay Bryant Endowed Professor, Department of Pathology, University of Michigan Medical School, A. Alfred Taubman Biomedical Science Research Building, 109 Zina Pitcher Place, BSRB 2037, Ann Arbor, MI 48109-2200, Tel.: 734-615-4388, Fax: 734-615-9666, E-mail: kojonen@umich.edu; Megan S. Lim, MD, PhD, Professor, Department of Pathology, University of Michigan, 109 Zina Pitcher Place, BSRB 2039, Ann Arbor, MI 48109-2200, Tel.: 734-615-4388, Fax: 734-615-9666, E-mail: meganlim@med.umich.edu.

REFERENCES

1. Rowley, J. D. (2001) Chromosome translocations: dangerous liaisons revisited. Nat. Rev. Cancer 1, 245–250
2. Stratton, M. R., Campbell, P. J., and Futreal, P. A. (2009) The cancer genome. Nature 458, 719–724
3. Mitelman, F., Johansson, B., and Mertens, F. (2007) The impact of translocations and gene fusions on cancer causation. Nat. Rev. Cancer 7, 233–245
4. Druker, B. J., Tamura, S., Buchdunger, E., Ohno, S., Segal, G. M., Fanning, S., Zimmermann, J., and Lydon, N. B. (1996) Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat. Med. 2, 561–566
5. Druker, B. J., Talpaz, M., Resta, D. J., Peng, B., Buchdunger, E., Ford, J. M., Lydon, N. B., Kantarjian, H., Capdeville, R., Ohno-Jones, S., and Sawyers, C. L. (2001) Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N. Engl. J. Med. 344, 1031–1037
6. Kwak, E. L., Bang, Y. J., Camidge, D. R., Shaw, A. T., Solomon, B., Maki, R. G., Ou, S. H., Dezube, B. J., Janne, P. A., Costa, D. B., Varella-Garcia, M., Kim, W. H., Lynch, T. J., Fidias, P., Stubbs, H., Engelman, J. A., Sequist, L. V., Tan, W., Gandhi, L., Mino-Kenudson, M., Wei, G. C., Shreeve, S. M., Ratain, M. J., Settleman, J., Christensen, J. G., Haber, D. A., Wilner, K., Salgia, R., Shapiro, G. I., Clark, J. W., and Iafarte, A. J. (2010) Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. N. Engl. J. Med. 363, 1693–1703
7. Doman, B., and Aebersold, R. (2006) Mass spectrometry and protein analysis. Science 312, 212–217
8. Yates, J. R., Ruse, C. I., and Nakorchevsky, A. (2009) Proteomics by mass spectrometry: approaches, advances, and applications. Annu. Rev. Biomed. Eng. 11, 49–79
9. Picotti, P., and Aebersold, R. (2012) Selected reaction monitoring-based proteomics: workflows, potential, pitfalls and future directions. Nat. Methods 9, 555–566
10. Anderson, L., and Hunter, C. L. (2006) Quantitative mass spectrometry of multiple reaction monitoring assays for major plasma proteins. Mol. Cell. Proteomics 5, 573–588
11. Keshishian, H., Addona, T., Burgess, M., Kuhn, E., and Carr, S. A. (2007) Quantitative, multiplexed assays for low abundance proteins in plasma by targeted mass spectrometry and stable isotope dilution. Mol. Cell. Proteomics 6, 2212–2229
12. Geoghegan, K. F., Hoth, L. R., Tan, D. H., Borzillesi, K. A., Witkha, J. M., and Boyd, J. G. (2002) Cyclization of N-terminal S-carbamoylmethylcysteine causing loss of 17 Da from peptides and extra peaks in peptide maps. J. Proteome Res. 1, 181–187
13. Keshishian, H., Addona, T., Burgess, M., Mani, D. R., Shi, X., Kuhn, E., Sabatine, M. S., and Carr, S. A. (2009) Quantification of cardiovascular biomarkers in patient plasma by targeted mass spectrometry and stable isotope dilution. Mol. Cell. Proteomics 8, 2339–2349
14. Morris, S. W., Kirstein, M. N., Valentine, M. B., Dittmer, K. G., Shapiro, D. N., Saltman, D. L., and Look, A. T. (1994) Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin’s lymphoma. Science 263,
15. Carr, S. A., and Anderson, L. (2008) Protein quantitation through targeted mass spectrometry: the way out of biomarker purgatory? *Clin. Chem.* **54**, 1749–1752

16. Wang, Q., Chaerkady, R., Wu, J., Hwang, H. J., Papadopoulos, N., Kopelowich, L., Maitra, A., Matthaei, H., Eshleman, J. R., Hruban, R. H., Kinzler, K. W., Pandey, A., and Vogelstein, B. (2011) Mutant proteins as cancer-specific biomarkers. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 2444–2449

17. Kuzyk, M. A., Smith, D., Yang, J., Cross, T. J., Jackson, A. M., Hardie, D. B., Anderson, N. L., and Borchers, C. H. (2009) Multiple reaction monitoring-based, multiplexed, absolute quantitation of 45 proteins in human plasma. *Mol. Cell. Proteomics* **8**, 1860–1877