Highly multiplexed quantifications of 299 somatic mutations in colorectal cancer patients by automated MALDI-TOF mass spectrometry

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Abstract

Background
Detection of somatic mutations in tumor tissues helps to understand tumor biology and guide treatment selection. Methods such as quantitative PCR can analyze a few mutations with high efficiency, while next generation sequencing (NGS) based methods can analyze hundreds to thousands of mutations. However, there is a lack of cost-effective method for quantitatively analyzing tens to a few hundred mutations of potential biological and clinical significance.

Methods
Through a comprehensive database and literature review we selected 299 mutations associated with colorectal cancer. We then designed a highly multiplexed assay panel (8-wells covering 299 mutations) based on an automated MADLI-TOF mass spectrometry (MS) platform. The multiplex panel was tested with a total of 319 freshly frozen tissues and 92 FFPE samples from 229 colorectal cancer patients, with 13 samples also analyzed by a targeted NGS method covering 532 genes.

Results
Multiplex somatic mutation panel based on MALDI-TOF MS detected and quantified at least one somatic mutation in 142 patients, with KRAS, TP53 and APC being the most frequently mutated genes. Extensive validation by both capillary sequencing and targeted NGS demonstrated high accuracy of the multiplex MS assay. Out of 35 mutations tested with plasmid constructs, sensitivities of 5% and 10% mutant allele frequency were achieved for 19 and 16 mutations, respectively.

Conclusions
Automated MALDI-TOF MS offers an efficient and cost-effective platform for highly multiplexed quantitation of 299 somatic mutations, which may be useful in studying the
biological and clinical significance of somatic mutations with large numbers of cancer tissues.

Background

The cancer genomes carry many different genomic alterations including somatic mutations. Most notably, driver mutations are causally implicated in oncogenesis. They have conferred a growth advantage on the cancer cells[1]. Increasing number of mutations are being characterized for better treatment selection and prognosis[2]. In non-small cell lung cancer, for example, patients with epidermal growth factor receptor (EGFR) mutation are likely to benefit from tyrosine kinase inhibitors (TKIs)[3]. On the opposite, colorectal cancer patients with KRAS or NRAS mutations should not be treated with either EGFR-inhibitor Cetuximab[4] or Panitumumab[5].

Several methods are available for the detection of tumor somatic mutations. Next generation sequencing (NGS) is widely adopted as a discovery tool for somatic mutation analysis in cancer[6]. Using NGS, The Cancer Genome Atlas (TCGA) presents genomic alternations identified from over 11,000 tumor samples from 33 cancer types, providing valuable insights into new targets for drug development, treatment selection, or even combination therapies for personalized medicine[7]. While NGS methods can analyze hundreds to tens of thousands mutations[8], methods such as qPCR or digital PCR are ideal for analyzing a few clinically important mutations with high efficiency and cost-effectiveness[9, 10]. However, as biologically and clinically important mutations for many cancer types continue to be identified, there is a growing unmet need to cost-effectively and quantitatively analyze tens to a few hundred of mutations, in highly heterogenous cancer tissues where mutant allele frequency can be highly variable.

The MassARRAY platform, based on automated MALDI-TOF mass spectrometry, is suitable to meet this growing need due to its high multiplex capability, flexibility for both sample
size and mutation number, quantification capability, and automation in sample processing
and data analysis[11]. The MassARRAY can detect up to 40 mutations in a single well on a
96 or 384-well plate.

In this report, we developed and validated an 8-well panel covering 299 most common
somatic mutations in colorectal cancer (CRC). Such multiplex level may be sufficient for
detecting virtually all clinically relevant mutations in a given cancer type, with cost-
effectiveness and turnaround time desirable in real-life clinical settings.

Methods

Patients recruitment

Patients diagnosed with colorectal cancer were recruited with informed consent between
July 2015 and June 2019 from the First Affiliated Hospital and the Second Affiliated
Hospital of Wenzhou Medical University, China. Exclusion criteria include more than two
pathological types, metastasis, treated with neoadjuvant chemotherapy or immunotherapy
before surgery, or any cancer within the past 5 years. Tumor stages were determined
according to the 8th edition of the American Joint Committee on Cancer (AJCC). The study
was approved by the ethics committee of Wenzhou Medical University and its affiliated
hospital.

Sample collection and DNA extraction

Resections or biopsies of primary solid tumors and adjacent tissues located 2-cm away
from the tumor tissue, were taken immediately after surgery, snap frozen in liquid
nitrogen, and stored at -80°C. DNA was extracted from tissue using the QIAamp DNA mini
kit (Qiagen, Hilden, Germany) according to the manufacturer’s instruction, and stored at
-20°C for further use. The DNA concentration was quantified by NanoDrop One (Thermo
Fisher Scientific, Foster City, CA, USA).
FFPE tumor samples were analyzed by with hematoxylin-eosin (HE) staining to determine tumor purity. For each FFPE tissue, 20 slices of 10-μm-thick sections were used for DNA extraction with the QIAamp DNA FFPE Tissue kit (Qiagen). Concentration of DNA was determined by a Qubit 3.0 Fluorometer (Thermo Fisher Scientific).

CRC mutation panel design

The mutations were selected for either of the following three reasons: 1) present in at least two of the following three sources for CRC samples: TCGA (https://cancergenome.nih.gov/); International Cancer Genome Consortium (ICGC) database (https://icgc.org/); and publication by Muzny DM et al.[12]; 2) potential resistance to CRC targeted therapy based on My Cancer Genome (http://www.mycancergenome.org), or 3) reported in a commercial cancer panel[13].

The CRC mutation panel was designed with Assay Designer software (MassARRAY Typer, Version 4.0, Agena Biosciences, San Diego, CA, USA), with primer sequences not overlapping known single nucleotide polymorphisms whenever possible.

CRC mutation detection and quantification by MALDI-TOF MS

CRC Mutation detection was optimized and performed by MassARRAY Analyzer 4 with CPM (Agena Biosciences). All primers were purchased from Integrated DNA Technologies (Integrated DNA Technologies, Coralville, IA, USA). All reagents were purchased from Agena Bioscience unless otherwise specified. Briefly, tissue genomic DNA was amplified by multiplex PCR. Shrimp alkaline phosphatase treatment was performed to inactivate surplus nucleotides. A primer extension reaction (iPLEX Pro) was performed with mass-modified terminator nucleotides, and the product was spotted on SpectroCHIP. Wild-type and mutant alleles were then discriminated by molecular weights determined by
MassARRAY analyzer.

Allele calls were performed with MassARRAY Typer Analyzer software (Typer 4.0.26). Additionally, at least two investigators independently reviewed the mass spectra to further confirm the automated calls by the software. To estimate mutant allele frequency, the heights of raw spectral peaks corresponding to the wild-type and mutation allele were quantified. Mutation allele frequency was estimated by calculating mutant peak / (mutant peak + wild type peak).

Targeted NGS and data analysis

Genomic DNA was fragmented to an average size of 200 to 500 bp using Bioruptor Pico sonication device (Diagenode, Denville, NJ, USA). Sequencing libraries were prepared using the KAPA LTP Library Preparation Kit for Illumina (Kapa Biosystems, Wilmington, MA, USA) according to manufacturer’s suggestions. Hybridization-based target enrichment was carried out with xGen Pan-Cancer Panel v2.4 (532 cancer-relevant genes), and xGen Lockdown Hybridization and Wash Reagents Kit (Integrated DNA Technologies). Captured libraries were amplified and purified using Agencourt AMPure XP Beads (Beckman Coulter, Atlanta, GA, USA). Concentrations and qualities of DNA libraries were analyzed by the Agilent High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA).

The libraries were paired-end sequenced on the Illumina HiSeq X platform (Illumina, San Diego, CA, USA) according to the manufacturer’s instructions. Sequencing adapters and low quality bases were trimmed from raw sequencing reads using Trim Galore (v0.4.1; https://github.com/FelixKrueger/TrimGalore). The filtered reads were then mapped to the reference Human Genome (hg38) using BWA-MEM (v0.7.12; https://github.com/lh3/bwa/tree/master/bwakit) with the default settings. The GATK (v4.0.12.0; https://software.broadinstitute.org/gatk/) was used for single nucleotide
variation (SNV) identifications. SNV calls with at least 2.5% variant allele frequency (VAF) were retained, followed by annotation using ANNOVAR [14].

Mutation validation by capillary sequencing

A subset of samples were selected for Sanger sequencing to validate MS results. The capillary sequencing was performed with the BigDye Terminator Cycle Sequencing Kit and ABI 3730 Genetic Analyzer (Thermo Fisher Scientific).

Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics 20.0. The $\chi^2$ test or Fisher exact test were used to compare baseline categorical variables, and the Kruskal-Wallis test was used to analyze the association between mutation and tumor size. The Cox proportional-hazards model of multivariate analysis was performed to analyze covariables, such as age, gender, clinical stage, location, differentiation, metastasis, treatment after surgery, and tumor size.

Results

Patients information

A total of 300 patients diagnosed with colorectal cancer between July 2015 and June 2019 were recruited. Seventy one patients were excluded (see exclusion criteria in the methods section). Frozen tissues (paired tumor and adjacent normal) from 136 patients, frozen tumor tissues from 47 patients, and formalin fixed paraffin embedded (FFPE) tissues (paired tumor and adjacent normal) from 46 patients with CRC were analyzed. Table 1 summarizes the patients’ baseline characteristics, tumor stage, location, differentiation, size, follow-up treatment after surgery and tumor marker (CEA) level.
Selection of somatic mutations and multiplex assay design

Somatic mutations for CRC were chosen based on their prevalence, and biological/clinical relevance. The final mutation panel consists of 299 mutations from 109 genes (Additional file 1: Supplementary Table 1).

We designed a 8-well multiple assay for a panel of 299 mutations (36-plex in well 1 and 2, 34-plex in well 3 and 5, 30-plex in well 4, 29-plex in well 6, 26-plex in well 7, and 12-plex in well 8) for MALDI-TOF MS analysis. The sequences of PCR primers and extension primers are provided in Additional file 1: Supplementary Table 2. Wild-type and mutant alleles were then discriminated by molecular weights of the extension products measured by MALDI-TOF MS (example results shown in Figure 1).

To determine the sensitivity of the assays, we constructed plasmids for 35 different mutations. Plasmid mutations were confirmed by Sanger sequencing. Mixtures of plasmids containing wild-type and mutant sequences to mimic samples with 5% and 10% mutation frequencies were prepared to analyze the sensitivity and accuracy of the assay. Among them, 19 assays achieved a 5% sensitivity for mutations, and 16 assays achieved a 10% sensitivity (Figure 2 and Supplement figure 1).

Mutation profiles of 229 patients from frozen and FFPE tissues

Using the multiplex assay, we analyzed 411 samples from 229 patients (319 freshly frozen tissues from 183 patients and 92 FFPE samples from 46 patients) (Figure 3). In the frozen tissue cohort, we identified and quantified 52 different somatic mutations in 107 patients. The most frequently mutated genes were KRAS (52 patients, 28.4%), TP53 (48 patients, 26.2%) and APC (32 patients, 17.5%). We chose 20 mutations from 31 patients for Sanger sequencing validation. Sanger Sequencing showed complete concordance with MALDI-TOF
MS (Additional file 2: Supplementary Figure 2).

In the cohort with 46 paired FFPE tissues (tumor and surrounding normal tissues), we first performed histopathological analysis with H&E staining (Supplement figure 3). With the multiplex MS assay, we detected a total of 39 mutations in 35 samples. Similarly, APC, TP53, and KRAS were most frequently mutated. We chose 15 different mutations (19 total mutations) from 13 patients for Sanger sequencing validation (Additional file 2: Supplementary Figure 4). Eighteen of the 19 mutations (95%) were validated successfully. Samples chosen for validation, as well as sequences of PCR primers and sequencing primers, are provided in Additional file 1: Supplementary Table 3.

We next examined the association between the molecular alterations and patient characteristics (Table 2). Among the 142 patients with at least one somatic mutation identified, we found that the 63 patients without a RAS mutation exhibited different clinical stage distribution as compared with the 79 patients with RAS mutation (p=0.019, $\chi^2$ test). TP53 mutations were more frequently found in male patients by univariate analysis (p=0.002, $\chi^2$ test).

Comparison between targeted NGS and MS assays

We chose 13 patient samples and performed targeted NGS sequencing using xGen Pan-Cancer Panel V2.4. The NGS panel covers 532 genes. Deep sequencing was performed to have an average sequencing depth of 1400. Among these 13 patients, the MS method identified 11 somatic mutations. Targeted NGS detected 14 mutations, including the 11 mutations detected by MS. Three mutations were identified only by NGS. These three discordant mutations were further analyzed by DNA cloning and capillary sequencing. Two mutations (KRAS_c.G35A, APC_c.C2626T) were verified by cloning and sequencing. One
mutation (KRAS_c.G34A) was present at 3% by targeted NGS. We sequenced 37 clones and did not observe any mutant.

Discussion

We developed an 8-well, multiplexed assay based on automated MALDI-TOF mass spectrometry to detect 299 CRC related mutations in 229 southern China patients. The selected 299 mutations cover the National Comprehensive Cancer Network (NCCN) guideline of colon cancer recommended gene list relevant to treatment and prognosis, such as KRAS, NRAS, BRAF V600E mutations [15-17]. The MALDI-TOF MS assay is sensitive and quantitative in analyzing somatic mutations, with extensive validation by capillary sequencing, as well as a head-to-head comparison with a targeted NGS panel covering 532 genes.

NGS-based methods can detect known and unknown mutations while the MALDI-TOF MS assay is more suitable to detect pre-selected, functionally relevant known mutations. The cost of the multiplex MS assay (about USD 60/sample) is substantially lower than targeted NGS (about USD 400/sample). The MS approach is highly automated and suitable for high throughput analysis of larger sample size, making it suitable as a routine testing platform.

Conclusions

We have developed and validated a highly multiplexed assay for the quantification of 299 somatic mutations in colorectal cancer tissues, offering a tool for studying the biological and clinical significance of somatic mutations with large numbers of cancer tissues. The multiplex assay may also be useful in clinical management of colorectal cancer patients.

Abbreviations

MALDI-TOF MS: matrix-assisted laser desorption/ionization-time of flight mass
spectrometry

PCR: polymerase chain reaction

qPCR: quantitative PCR

**KRAS**: KRAS proto-oncogene, GTPase

**TP53**: tumor protein p53

**APC**: APC, WNT signaling pathway regulator

**EGFR**: epidermal growth factor receptor

**TKI**: tyrosine kinase inhibitor

**NRAS**: NRAS proto-oncogene, GTPase

**NGS**: next-generation sequencing

**TCGA**: The Cancer Genome Atlas

**CRC**: colorectal cancer

**AJCC**: American Joint Committee on Cancer

**HE**: Hematoxylin-Eosin

**ICGC**: International Cancer Genome Consortium

**SNV**: Single Nucleotide Variation

**SNPs**: Single Nucleotide Polymorphisms

**VAF**: Variant Allele Frequency

**CEA**: Carcinoembryonic Antigen

**TCF7L2**: transcription factor 7 like 2

**PFS**: progress-free survival

**NCCN**: National Comprehensive Cancer Network

**BRAF**: B-Raf Proto-Oncogene, Serine/Threonine Kinase

**Declarations**

*Ethics approval and consent to participate*
The study was approved by the Clinical Research Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University and Wenzhou Medical University (ref. no. 2016-161). Written informed consent was obtained from participants.

**Consent for publication**

Not applicable

**Availability of data and material**

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

CD, SJ and CX conceived, designed, supervised the study and critically revised the manuscript. CX, DP, JL, MC, YH, MH, QS, QL, JL, WL, XW, CL, WH, MC, HZ, GC, LY, XZ, and
FJ provided biospecimens and clinical data. JL, DP, JL, MH and QL performed the laboratory work. JL and DP performed data analysis. All authors read and approved the final manuscript.

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Tables

Table 1. Patient characteristics.

| Characteristic | All patients (N=229) (%) | Frozen tissue cohort (N=183) (%) | FFPE tissue cohort (N=46) (%) |
|----------------|--------------------------|---------------------------------|-----------------------------|
| Age - no.      |                          |                                 |                             |
| <50            | 20 (8.7)                 | 17 (9.3)                        | 3 (6.5)                     |
| 50-70          | 124 (54.1)               | 101 (55.2)                      | 23 (50.0)                   |
| >70            | 85 (37.2)                | 65 (35.3)                       | 20 (43.5)                   |

| Gender - no.  |                          |                                 |                             |
| Male          | 145 (63.3)               | 113 (61.7)                      | 32 (69.6)                   |
| Female        | 84 (36.7)                | 70 (38.3)                       | 14 (30.4)                   |
### Clinical stage - no.

| Stage | No. 53 (23.2) | No. 34 (18.6) | No. 19 (41.3) |
|-------|---------------|---------------|---------------|
| I     | 69 (30.1)     | 59 (32.3)     | 10 (21.7)     |
| II    | 82 (35.8)     | 71 (38.8)     | 11 (24.0)     |
| III   | 25 (10.9)     | 19 (10.4)     | 6 (13.0)      |

### Location sampled - no.

| Location                     | No. 177 (77.3) | No. 140 (76.5) | No. 37 (80.5) |
|------------------------------|----------------|----------------|---------------|
| Rectum                      | 19 (8.3)       | 17 (9.3)       | 2 (4.3)       |
| Left Colon                  | 21 (9.2)       | 16 (8.7)       | 5 (10.9)      |
| Rectosigmoid Junction       | 12 (5.2)       | 10 (5.5)       | 2 (4.3)       |

### Differentiation - no.

| Differentiation | No. 13 (5.7) | No. 12 (6.6) | No. 1 (2.2) |
|-----------------|--------------|--------------|-------------|
| Well            | 180 (78.6)   | 145 (79.2)   | 35 (76.0)   |
| Poor            | 28 (12.2)    | 19 (10.4)    | 9 (19.6)    |
| Unknown†        | 8 (3.5)      | 7 (3.8)      | 1 (2.2)     |
### Metastatic sites - no.

|                | No.   | No.   | No.   |
|----------------|-------|-------|-------|
| Liver only     | 19 (8.3) | 15 (8.2) | 4 (8.7) |
| Lung only      | 2 (0.9)  | 1 (0.5)  | 1 (2.2)  |
| Liver and Lung | 1 (0.4)  | 1 (0.5)  | 0 (0.0)  |
| Bladder        | 1 (0.4)  | 1 (0.5)  | 0 (0.0)  |
| Brain          | 1 (0.4)  | 1 (0.5)  | 0 (0.0)  |

### Treatment after surgery - no.

|                      | No.   | No.   | No.   |
|----------------------|-------|-------|-------|
| With adjuvant therapy| 129 (56.3) | 109 (59.6) | 20 (43.5) |
| Without adjuvant therapy | 96 (42.0) | 74 (40.4) | 22 (47.8) |
| NA                   | 4 (1.7)  | 0 (0.0)  | 4 (8.7)  |

### Tumor size - cm

|                | Median (range) |
|----------------|----------------|
| No. 1          | 4(1.5-13)      |
| No. 2          | 4 (1.5-13)     |
| No. 3          | 4(1.5-12)      |

### CEA - ng/mL

|        | No.   | No.   | No.   |
|--------|-------|-------|-------|
| 0.0-5.0| 150 (65.5) | 125 (68.3) | 25 (54.3) |
| >5.0   | 74 (32.3)  | 53 (29.0)  | 21 (45.6)  |
† Reported as mucinous adenocarcinomas.

N/A: not available

Table 2. Patient characteristics in different molecular subgroups.

|                  | With any mutation | No mutati on | P value | With AP C mutation | With other mutation but no AP C mutation | P value | With TP53 mutation | With other mutation but no TP53 mutation | P value | With RA S mutation | With other mutation but no RA S mutation | P value |
|------------------|-------------------|--------------|---------|--------------------|----------------------------------------|---------|-------------------|----------------------------------------|---------|-------------------|----------------------------------------|---------|
| Patients - no.   | 142               | 87           |         | 41                 | 101                                    |         | 66                | 76                                     |         | 79                | 63                                     |         |
| Age Media (yr)   | 66.0              | 65.3         |         | 64                 | 67                                     |         | 65                | 67                                     |         | 65                | 67                                     |         |
| (range)          | (40-90)           | (31-87)      |         | (42-90)            | (40-82)                                |         | (44-90)           | (40-90)                               |         | (42-88)           | (40-90)                               |         |
| <50 no.          | 11                | 9            | 0.5     | 5                  | 6                                      | 0.8     | 5                 | 6                                      | 0.1     | 5                 | 6                                      | 0.5     |
| 50-70 no.        | 77                | 47           |         | 21                 | 56                                     |         | 40                | 37                                     |         | 46                | 31                                     |         |
| >70 no.          | 54                | 31           |         | 15                 | 39                                     |         | 21                | 33                                     |         | 28                | 26                                     |         |
| Gender - no.     | 0.2               | 0.5          |         | 0.8               | 0.0                                    |         | 0.0               | 0.0                                    |         | 0.0               | 0.0                                    |         |
| Male             | 85                | 60           |         | 24                 | 61                                     |         | 49                | 36                                     |         | 43                | 42                                     |         |
| Female           | 57                | 27           |         | 17                 | 40                                     |         | 17                | 40                                     |         | 36                | 21                                     |         |
| Clinical stage - no. | 0.0 | 0.6               |         | 0.2               | 0.0                                    |         | 0.0               | 0.0                                    |         | 0.0               | 0.0                                    |         |
|     |     |     |     |
|-----|-----|-----|-----|
| I   | 35  | 18  | 21  |
| II  | 37  | 32  | 12  |
| III | 49  | 33  | 11  |
| IV  | 21  | 4   | 6   |

**Location sampled - no.**

|     |     |     |     |
|-----|-----|-----|-----|
|     | 0.0 | 0.2 | 0.2 |
|     | 62  | 66  | 13  |

**Rectum**

|     |     |     |     |
|-----|-----|-----|-----|
|     | 117 | 60  | 57  |
|     | 35  | 82  | 60  |
|     | 70  | 47  |     |

**Left Colon**

|     |     |     |     |
|-----|-----|-----|-----|
|     | 8   | 11  | 2   |
|     | 1   | 7   | 6   |
|     | 2   | 6   |     |

**Right Colon**

|     |     |     |     |
|-----|-----|-----|-----|
|     | 9   | 12  | 1   |
|     | 2   | 7   |     |
|     | 3   | 6   |     |

**Rectosigmoid Junction**

|     |     |     |     |
|-----|-----|-----|-----|
|     | 8   | 4   | 5   |
|     | 4   | 4   | 3   |
|     | 4   | 4   |     |

**Differentiation - no.**

|     |     |     |     |
|-----|-----|-----|-----|
|     | 0.8 | 0.1 | 0.1 |
|     | 92  | 28  | 56  |
|     |     |     | 0.4 |

**Well**

|     |     |     |     |
|-----|-----|-----|-----|
|     | 8   | 5   | 0   |
|     | 5   | 3   | 6   |
|     | 2   |     |     |

**Moderate**

|     |     |     |     |
|-----|-----|-----|-----|
|     | 113 | 67  | 36  |
|     | 50  | 63  | 60  |
|     | 53  |     |     |

**Poor**

|     |     |     |     |
|-----|-----|-----|-----|
|     | 16  | 12  | 3   |
|     | 13  | 11  | 5   |
|     | 10  | 6   |     |

**Unknown†**

|     |     |     |     |
|-----|-----|-----|-----|
|     | 5   | 3   | 2   |
|     | 2   | 3   | 0   |
|     | 3   | 2   |     |

**Metastasis - no.**

|     |     |     |     |
|-----|-----|-----|-----|
|     | 0.0 | 1.0 | 0.8 |
|     | 17  | 0.0 | 15  |
|     | 14  |     |     |

**With**

|     |     |     |     |
|-----|-----|-----|-----|
|     | 21  | 4   | 6   |
|     | 15  | 9   | 12  |
|     | 11  | 10  |     |

**Without**

|     |     |     |     |
|-----|-----|-----|-----|
|     | 121 | 83  | 35  |
|     | 56  | 64  | 68  |
|     | 53  |     |     |

**Treatment after surgery - no.**

|     |     |     |     |
|-----|-----|-----|-----|
|     | 0.4 | 0.1 | 0.6 |
|     | 0.5 | 0.3 | 0.6 |
|     | 1.0 |     |     |

**With adjuvant therapy**

|     |     |     |     |
|-----|-----|-----|-----|
|     | 77  | 52  | 26  |
|     | 51  | 34  | 43  |
|     |     | 42  | 35  |
Wit- out  
adj- u- n- 
the  
rap-  
N/A  2  2  1  1  0  0  1  1  0  5  2  0  0  4  3  2  1  32  66  98  79  0.3  
Tumor 
siz- 
ecm  
Me- 
dia- 
(1.5  (1.5  (1.5  (1.5  (1.5  (1.5  (1.5  (1.5  (1.5  (1.5  8.5)  
(13)  (10)  
(13)  (10)  
(13)  (10)  
(13)  (10)  
(13)  (10)  
(13)  (10)  
Me- 
an  
C- 
A- 
ng/  
mL  
0.0-  
5.0  
>5. 
0  
N/A  2  3  1  1  0  2  0  2  
P values were calculated with the use of $\chi^2$ test or Fisher exact test for categorical variables and Kruskal-Wallis test for continuous variables. 
† Reported as mucinous adenocarcinomas. 
N/A: not available
Figure 1

Representative MS results showing paired tumor and adjacent tissues from frozen and FFPE tissue cohort. Shown in (A), (B), and (C) are three different mutations in the TP53, APC and KRAS genes from frozen tissue cohort, (D) and (E) shows the mass spectrum of the same site as the frozen tissues, respectively. Although (F) and (C) are not the same site, they belong to an assay. Mutant allele frequency can be estimated by comparing the peak signals for the mutant and wild type alleles. Wt: wild type, Mut: mutation.
Evaluation performance of MS assay. The DNA mixture samples with 5\% and 10\% mutation were prepared to analyze the sensitivity and accuracy of the assay. Among them, 19 assays achieved a 5\% sensitivity for mutations, and 16 assays achieved a 10\% sensitivity. Here we show 4 assays that could achieve a 5\% sensitivity.

Mutation profiles quantified by the multiplex CRC panel. Each colored bar represents one mutation (orange) or two mutations in the same gene (blue). The heights of the colored bars represent mutant allele frequencies.
Supplementary Files

This is a list of supplementary files associated with the primary manuscript. Click to download.

Additional file 1.xls
Additional file 2.pdf