BMJ Open

Validation of targeted next-generation sequencing for *RAS* mutation detection in FFPE colorectal cancer tissues: comparison with Sanger sequencing and ARMS-Scorpion real-time PCR

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ABSTRACT

Objective: To validate the targeted next-generation sequencing (NGS) platform-Ion Torrent PGM for *KRAS* exon 2 and expanded *RAS* mutations detection in formalin-fixed paraffin-embedded (FFPE) colorectal cancer (CRC) specimens, with comparison of Sanger sequencing and ARMS-Scorpion real-time PCR.

Setting: Beijing, China.

Participants: 51 archived FFPE CRC samples (36 men, 15 women) were retrospectively randomly selected and then checked by an experienced pathologist for sequencing based on histological confirmation of CRC and availability of sufficient tissue.

Methods: RAS mutations were detected in the 51 FFPE CRC samples by PGM analysis, Sanger sequencing and the Therascreen *KRAS* assay, respectively. Agreement among the 3 methods was assessed. Assay sensitivity was further determined by sequencing serially diluted DNA from FFPE cell lines with known mutation statuses.

Results: 13 of 51 (25.5%) cases had a mutation in *KRAS* exon 2, as determined by PGM analysis. PGM analysis showed 100% (51/51) concordance with Sanger sequencing ($\kappa=1.000$, 95% CI 1 to 1) and 98.04% (50/51) agreement with the Therascreen assay ($\kappa=0.947$, 95% CI 0.844 to 1) for detecting *KRAS* exon 2 mutations, respectively. The only discrepant case harboured a *KRAS* exon 2 mutation (c.37G>T) that was not covered by the Therascreen kit. The dilution series experiment results showed that PGM was able to detect *KRAS* mutations at a frequency of as low as 1%. Importantly, *RAS* mutations other than *KRAS* exon 2 mutations were also detected in 10 samples by PGM. Furthermore, mutations in other CRC-related genes could be simultaneously detected in a single test by PGM.

Conclusions: The targeted NGS platform is specific and sensitive for *KRAS* exon 2 mutation detection and is appropriate for use in routine clinical testing. Moreover, it is sample saving and cost-efficient and time-efficient, and has great potential for clinical application to expand testing to include mutations in *RAS* and other CRC-related genes.

Strengths and limitations of this study

- This is the first study to evaluate the targeted next-generation sequencing (NGS) platform-Ion Torrent PGM and Ion AmpliSeq Cancer Hotspot Panel for expanded *RAS* mutation testing in formalin-fixed paraffin-embedded (FFPE) colorectal cancer (CRC) specimens.
- Our results highlight the strengths of the targeted NGS platform-Ion Torrent PGM for *KRAS* exon 2 and expanded *RAS* mutation detection in FFPE CRC specimens, with comparison of Sanger sequencing and ARMS-Scorpion real-time PCR.
- The study is limited by the relatively small sample size.

INTRODUCTION

We are approaching an era of personalised and precision medicine where cancer diagnosis and treatment will be tailored to each individual patient depending on their unique genetic signatures. Epidermal growth factor receptor (EGFR) plays important roles in the growth and survival of many solid cancers, including colorectal cancer (CRC).[1] Anti-EGFR therapies have been proven to be effective in a subset of patients with metastatic CRC. Given that only a small subgroup of patients with metastatic colon cancer respond to cetuximab, prediction of patient responses to anti-EGFR therapies is necessary to avoid side effects and to save costs.[2] RAS proteins (HRAS, KRAS and NRAS) are important downstream effectors that transmit signals from cell surface receptors, including EGFR, to intracellular signalling cascades by cycling from an inactive GDP-bound form to an active GTP-bound form. The *KRAS* gene, a member of the *RAS* family, is mutated in approximately

35–45% of CRC, and mutations in codons 12 and 13 of exon 2 of the *KRAS* gene render the GTPase insensitive to GAP stimulation, leading to the constitutive activation of this protein in the GTP-bound form.[3] It is well known that patients with *KRAS*-mutant CRC are resistant to treatment with anti-EGFR agents (cetuximab/panitumumab) because the *KRAS* activating mutation occurs downstream of EGFR. *KRAS* has been confirmed as a predictive biomarker for the efficacy of anti-EGFR therapy in CRC.[3] For this reason, *KRAS* mutation testing is being increasingly recommended for the selection of patients with CRC to receive anti-EGFR therapy.[4] [5] Testing for *KRAS* exon 2 mutations has been added to the National Comprehensive Cancer Network (NCCN) clinical practice guidelines for colon cancer since 2008. Moreover, extending *KRAS* exon 2 testing to include *RAS* has been recommended in the latest NCCN colon cancer guidelines (V.2.2015).

A number of sequencing and PCR-based techniques are currently available for *KRAS* mutation testing in clinical practice, and each has specific advantages and limitations.[2] [6] [7] Traditional Sanger sequencing has been considered the gold standard for identifying mutations for many years due to its low false-positive rate and high specificity. However, this method is limited by its low sensitivity, and it is also time consuming due to the need to manually analyse sequencing chromatograms to search for mutations. The Therascreen *KRAS* Mutation Kit (Qiagen, Hilden, Germany) has been developed for performing a real-time, allele-specific assay (PCR) using Scorpion ARMS technology to detect the most common *KRAS* mutations in codons 12 and 13. It has greater sensitivity for detecting low-level mutations compared with that of Sanger sequencing, and it has been Food and Drug Administration (FDA) approved for *KRAS* mutation detection using DNA extracted from formalin-fixed paraffin-embedded (FFPE) colorectal adenocarcinoma tissue.[8] However, the Therascreen assay was designed to only detect specific somatic mutations in exon 2 of the *KRAS* oncogene.[9] Moreover, the traditional methods are especially costly and time consuming for expanded *RAS* mutation or multiplex genetic testing.

In recent years, rapid technological advances have broadened next-generation sequencing (NGS) applications from research to clinical testing.[10] NGS provides a cost-effective, high-sensitivity and high-throughput platform for examining genetic aberrations in the clinical setting.[11] As we know, the recently updated NCCN guideline strongly recommends genotyping of tumour tissue (either primary tumour or metastasis) in all patients with metastatic CRC for *RAS* (*KRAS* exon 2 and non-exon 2, and *NRAS*), and patients with any known *KRAS* or *NRAS* mutation should not be treated with cetuximab or panitumumab. Given the sample-inefficiency, cost-inefficiency and time-inefficiency of traditional methods, the NGS platform has great potential for clinical application in performing such multiplex genetic testing in FFPE CRC specimens. Currently, several NGS platforms are commercially available for the sequencing of either targeted

genomic regions or whole genomes/exomes to analyse a variety of disease-associated genetic alterations, such as point mutations, insertions, deletions and copy number variations.[12] However, routine application of NGS in clinical oncology is still in its infancy.[11] Ion Torrent PGM is a promising NGS platform for targeted gene panel sequencing; it has demonstrated high productivity and performance, and shows clinical promise, especially for detecting single nucleotide variants (SNVs).[13–15] Although there are several other studies comparing NGS platforms and other methods, the potential clinical application of Ion Torrent PGM for determining *RAS* mutation status in FFPE CRC samples has not yet been well investigated, which is especially meaningful based on the latest NCCN CRC guidelines. Therefore, in this study, we aimed to analyse its performance for detection of *KRAS* exon 2 mutations in terms of sensitivity and specificity compared with two other methods that are currently used in clinical practice, bidirectional Sanger sequencing and the Therascreen *KRAS* assay, and to explore its potential clinical use for extended *RAS* mutation testing.

METHODS
Sample size calculation
Our sample size calculation was based on the following formula as described by Karimollah[16]:

$$n = \frac{Z_{\alpha/2}^2 \widehat{P}(1 - \widehat{P})}{d^2}$$

$\widehat{P}$ is the predetermined value of sensitivity (or specificity) that is ascertained by previous published data or clinician experience/judgement; α is the confidence level for statistical judgement where α is the probability of type I error; for α=0.05, $Z_{\alpha/2}$=1.96; d is the precision of estimates of sensitivity (or specificity), that is, the maximum difference between estimated sensitivity (or specificity) and the true value. As for our study, the predetermined values of sensitivity and specificity were 99% and 98%, respectively, $Z_{\alpha/2}$=1.96, and the margin of error (d) was set as ±5%, which yielded results that would be accurate to within ±5 percentage points. Based on the formula, the sample sizes for sensitivity and specificity were 15 and 30, respectively. Subsequently, the overall sample sizes for sensitivity and specificity were calculated using the following formulae, respectively:

$$n_{Se} = \frac{Z_{\alpha/2}^2 \widehat{Se}(1 - \widehat{Se})}{d^2 \times \text{Prev}}$$

$$n_{Sp} = \frac{Z_{\alpha/2}^2 \widehat{Sp}(1 - \widehat{Sp})}{d^2 \times \text{Prev}}$$

Prev denotes the prevalence of disease in the population. The prevalence of disease in the population was 40% in our present study, and thus the overall sample

sizes calculated based on sensitivity and specificity were 38.0 and 50.2, respectively. The maximum total number of participants based on sensitivity and specificity was 50.2, and thus a sample size of 51 was finally selected in our study.

Tissue samples and cell lines

A total of 51 archived FFPE CRC patients' samples (including 43 primary and 8 metastatic lesions) were retrospectively retrieved from surgical pathology files at the Peking Union Medical College Hospital from January 2014 to March 2015. Simple random sampling, based on random numbers generated by Microsoft Excel, was used to select the cases to avoid sample selection bias. These samples (36 surgical specimens and 15 biopsy specimens) were checked for sequencing by an experienced pathologist, based on histological confirmation of colorectal adenocarcinoma and availability of sufficient FFPE tumour tissue for performing the three methods. Patient's informed consent was obtained.

Pancreatic cell lines (Panc-1 and BxPC-3) were obtained from the ATCC. They were fixed and embedded using the same methods employed for the tissue samples.

DNA extraction

An appropriate paraffin block containing tumour tissue was selected for analysis by an experienced pathologist, who reviewed H&E-stained slides. The tumour area on the H&E-stained slide was marked and then manually macrodissected from up to 10 unstained sections (5 mm in thickness) to enrich for tumour cells before DNA extraction. Tumour cell content ranged from 60% to 95%, with a median cellularity of 80%. DNA was isolated using a QIAamp DNA Mini Kit (Qiagen). The same DNA extraction method was used for the FFPE cell lines. The extracted DNA was then quantified using a Qubit dsDNA BR assay (Life Technologies, Gaithersburg, USA).

Ion Torrent PGM sequencing and data processing

In total, 10 ng of DNA were used as template to generate an amplicon library for sequencing. Libraries were prepared using an Ion AmpliSeq Library Kit 2.0 and an Ion AmpliSeq Cancer Hotspot Panel v2 (Life Technologies), which amplifies 207 amplicons covering approximately 2800 COSMIC mutations in 50 oncogenes and tumour suppressor genes, with wide coverage of RAS (KRAS, NRAS and HRAS) and other CRC-related genes (BRAF, TP53, APC, MLH1, PIK3CA, SMAD4, FBWX7, etc). Adapter ligation, nick repair and PCR amplification were performed according to the manufacturer's protocol. The libraries were then quantified using a Qubit dsDNA HS Assay Kit and a Qubit 2.0 fluorometer (Life Technologies). Next, they were each diluted to a concentration of 3 ng/mL and pooled in equal volumes. Emulsion PCR and enrichment steps were carried out using an Ion OneTouch Template Kit and an Ion

OneTouch system (Life Technologies), according to the manufacturer's protocol. Following enrichment, the amplicon libraries were sequenced with an Ion Torrent PGM system (Life Technologies), using a 314 Chip, with barcoding performed using an Ion Xpress Barcode Adapter 1–16 Kit (Life Technologies). After sequencing, the readings were mapped to the reference genome (hg19) using the Torrent Mapping Alignment Program (TMAP). A cut-off of 300 000 AQ20 readings was used as a measure of successful sequencing on the 314 Chip. Variants were identified using Torrent Variant Caller (3.6.6; Life Technologies). A sequencing coverage of 200X was used as the minimum requirement to verify the authenticity of sequence variants. Integrative Genomics Viewer (Broad Institute, Massachusetts, USA) was used to visualise variants aligned against the reference genome to confirm the accuracy of the variant calls by checking for possible strand biases and sequencing errors.

ARMS/Scorpion real-time PCR

A Therascreen KRAS Mutation Kit (Qiagen) was used according to the manufacturer's instructions. This assay is designed to detect a wild-type control and the seven most common KRAS mutations in codons 12 and 13 of exon 2, as shown in table 1. Real-time PCR was performed on a Rotor-Gene Q Real-time PCR Platform (Qiagen). The cycling conditions for quality control runs and mutation assays were as follows: 15 min at 95°C, followed by 40 cycles at 95°C for 30 s and 60°C for 1 min. Fluorescence was measured at 60°C. Data on each mutation were interpreted according to the kit manual after curve analysis and calculation of ΔCt values.

Sanger sequencing

RAS mutations were detected by PCR-based 2× bidirectional direct Sanger sequencing using the primers specified in table 2. The sequencing results were interpreted using Chromas software V.1.45 (Technelysium Pty, Helensvale, Australia).

Assay sensitivity

The human pancreatic cancer cell line Panc-1 is known to harbour a heterozygous KRAS p.G12D (c.35G>A) mutation and a homozygous TP53 p.R273H (c.818G>A)

Table 1 KRAS mutations detected by the Therascreen KRAS Mutation Kit

Amino acid change	Base change	Cosmic ID
G12A	35G>C	522
G12D	35G>A	521
G12R	34G>C	518
G12C	34G>T	516
G12S	34G>A	517
G12V	35G>T	520
G13D	38G>A	532

Table 2 Details of the primers used for Sanger sequencing in the present study

Gene	Exon	Forward primer	Reverse primer
KRAS	2	GTGTGACATGTTCTAATATAGTCA	GAATGGTCCTGCACCAGTAA
KRAS	3	GGATTCCTACAGGAAGCAAGT	TGGCAAATACACAAAGAAAGC
KRAS	4	GGACTCTGAAGATGTACCTATGG	TCAACAAAGAAAGCCCTCCCCA
NRAS	2	ATGACTGAGTACAAACTGGT	CTCTATGGTGGGATCATATT
NRAS	3	GGTGAAACCTGTTTGTTGGA	AACCTAAAACCAACTCTTCCCA
NRAS	4	TTCCCGTTTTTAGGGAGCAGA	TGCAAACTCTTGCACAAATGC

mutation, whereas BxPC-3 has no mutations at these two sites. The assay sensitivity for SNV detection was determined by sequencing serially diluted DNA isolated from the two cell lines. FFPE Panc-1 DNA was mixed with FFPE BxPC-3 DNA at ratios of 1:0, 1:1, 1:4, 1:9, 1:19, 1:49 and 1:99 (Panc-1:BxPC-3), resulting in 100%, 50%, 20%, 10%, 5%, 2% and 1% dilutions, respectively.

Statistical analysis

The κ coefficient with 95% CI was used to assess agreement between the assay methods. Analyses were performed using SPSS software for windows, V.20 (SPSS Inc, Chicago, Illinois, USA).

RESULTS

Detection of *KRAS* exon 2 mutations in 51 FFPE CRC samples by three methods

A total of 51 samples were retrospectively collected from January 2014 to March 2015. The patients included 36 men and 15 women, with a median age of 63.0 years (range: 41–87 years). Of 51 samples, 13 (25.5%) had a mutation in exon 2 of *KRAS*, as determined by Ion Torrent PGM NGS, with coverage ranging from 369X to 2110X and the variant frequency ranging from 22.2 to 59.1% (table 3 and figure 1A–C). The number of samples with a *KRAS* exon 2 mutation detected using Sanger sequencing was 13/51 (25.5%), and it was 12/51

(23.5%) using the Therascreen assay. Ion Torrent PGM analysis showed 100% concordance with Sanger sequencing for *KRAS* exon 2 mutation testing (κ=1.000, 95% CI 1 to 1). High agreement between Ion Torrent PGM/Sanger sequencing and Therascreen real-time PCR was also demonstrated (κ=0.947, 95% CI 0.844 to 1), and only one case of discrepant results (1/51) was found between these methods (table 3 and figure 1D–F). This discrepant case (case 37) had a specific mutation (c.37G>T) that was not covered and thus could not be detected with a Therascreen *KRAS* Kit.

Assay sensitivity using FFPE cell lines

To further compare the sensitivities of Ion Torrent PGM and Sanger sequencing for SNV testing, we performed a dilution series experiment using FFPE human pancreatic cancer cell lines with known mutation statuses at two sites (*KRAS* codon 12 and *TP53* codon 273), as described in the Methods section. Our results showed that Ion Torrent PGM sequencing was able to detect the *KRAS* mutation (c.35G>A) at a level of as low as 1%, whereas the Sanger sequencing data were difficult to interpret when the dilutions (Panc-1: BxPC-3) fell below 10% (table 4, figures 2A–F and 3A–F). Similar results were obtained with *TP53* mutation (c.818G>A) detection (data not shown). Our results confirmed the greater sensitivity of Ion Torrent PGM compared with that of Sanger sequencing. Moreover,

Table 3 Detection of *KRAS* exon 2 mutations in 51 FFPE CRC samples by three methods

Patient No	Exon	Tumour cell percentage (%)	Ion torrent PGM Variants	Coverage (X)	Frequency (%)	Scorpions-ARMS Variants	Sanger Variants
2	2	65	p.G13D(c.38G>A)	546	38.5	p.G13D(c.38G>A)	p.G13D(c.38G>A)
3	2	85	p.G12D(c.35G>A)	2110	49.8	p.G12D(c.35G>A)	p.G12D(c.35G>A)
6	2	90	p.G12D(c.35G>A)	640	28.1	p.G12D(c.35G>A)	p.G12D(c.35G>A)
15	2	70	p.G12D(c.35G>A)	609	23.0	p.G12D(c.35G>A)	p.G12D(c.35G>A)
18	2	80	p.G12D(c.35G>A)	369	50.1	p.G12D(c.35G>A)	p.G12D(c.35G>A)
20	2	85	p.G12V(c.35G>T)	820	56.6	p.G12V(c.35G>T)	p.G12V(c.35G>T)
24	2	70	p.G12D(c.35G>A)	1035	54.2	p.G12D(c.35G>A)	p.G12D(c.35G>A)
25	2	70	p.G12D(c.35G>A)	1560	53.4	p.G12D(c.35G>A)	p.G12D(c.35G>A)
34	2	80	p.G12D(c.35G>A)	885	27.9	p.G12D(c.35G>A)	p.G12D(c.35G>A)
35	2	65	p.G13D(c.38G>A)	639	36.3	p.G13D(c.38G>A)	p.G13D(c.38G>A)
37	**2**	**90**	**p.G13C(c.37G>T)**	**628**	**59.1**	**ND**	**p.G13C(c.37G>T)**
42	2	75	p.G12D(c.35G>A)	1580	22.2	p.G12D(c.35G>A)	p.G12D(c.35G>A)
44	2	80	p.G12D(c.35G>A)	1728	31.7	p.G12D(c.35G>A)	p.G12D(c.35G>A)

Bold typeface indicates the discrepant case among three methods.
CRC, colorectal cancer; FFPE, formalin-fixed paraffin-embedded; ND, mutation status could not be determined by the Therascreen kit.
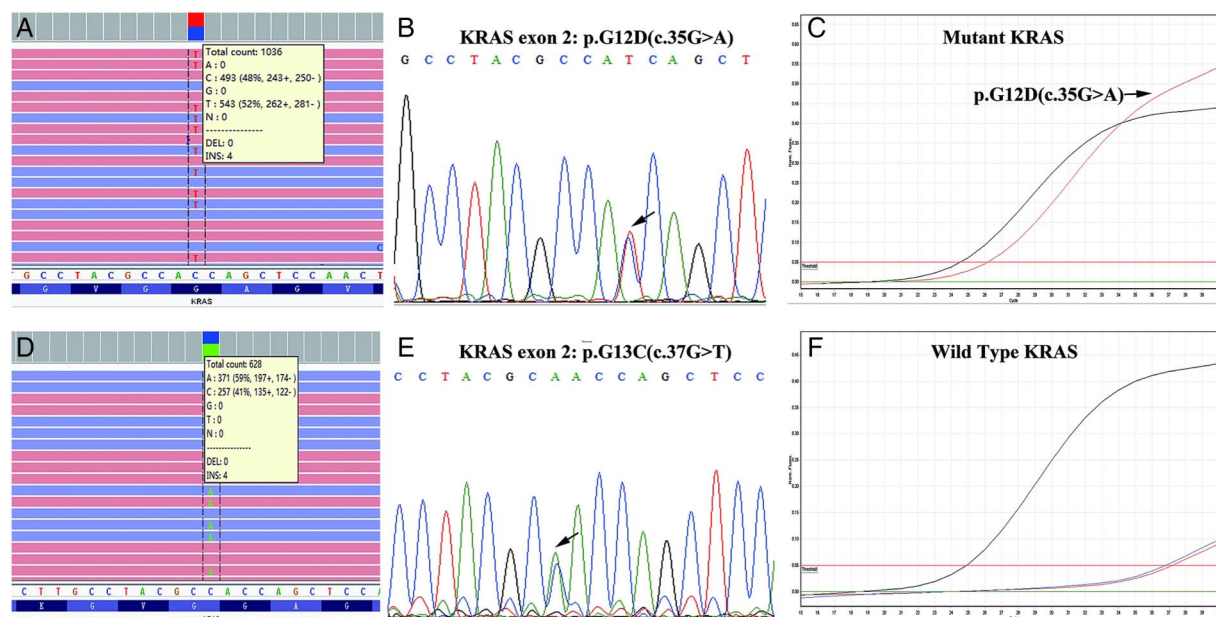
Figure 1 Detection of KRAS exon 2 mutations in case 24 (A–C) and case 37 (D–F) by Ion Torrent PGM, Sanger sequencing (reverse sequencing) and the Therascreen assay, respectively.

our results showed that Ion Torrent PGM could detect the *KRAS* mutation (c.35G>A) at various percentages, with excellent linearity (R^2=0.97) (figure 4A). There was also a strong correlation (R^2=0.97) between the dilutions (expected mutation frequencies) and calculated mutation frequencies for *TP53* mutation (c.818G>A) detection (figure 4B). However, when the dilutions/expected variant frequency fell below 5%, the correlation became weaker, likely due to baseline variation.

Potential application of Ion Torrent PGM for extended *RAS* analysis in FFPE CRC samples

We also explored the potential application of the Ion Torrent PGM platform for extended *RAS* analysis. A total of 10 cases had a *RAS* mutation other than a *KRAS* exon 2 mutation (non-exon 2 *KRAS* mutation, *NRAS*), with coverage ranging from 462X to 2420X and the variant frequency ranging from 14.3% to 66.4%,

accounting for 19.6% (10/51) of all cases and 26.3% (10/38) of *KRAS* exon 2 wild-type cases. Among these additional *RAS* mutations, three cases harboured a *KRAS* exon 3 mutation, six harboured a *KRAS* exon 4 mutation and one case harboured an *NRAS* exon 3 mutation, respectively (table 5). All of these mutations were confirmed by Sanger sequencing (figure 5A–F). However, detection of a low-frequency mutation by Sanger sequencing is relatively difficult (figure 6A, B).

Furthermore, mutations in other CRC-related genes (BRAF, *TP53*, APC, MLH1, PIK3CA, SMAD4, FBWX7, etc) were simultaneously detected by Ion Torrent PGM (see online supplementary appendix figure S1).

DISCUSSION

KRAS mutation status is critical when considering anti-EGFR therapy for patients with metastatic CRC. Although a number of methodologies are currently

Table 4 Sequencing of serially diluted DNA isolated from formalin-fixed paraffin-embedded (FFPE) human pancreatic cancer cell lines with known mutation statuses

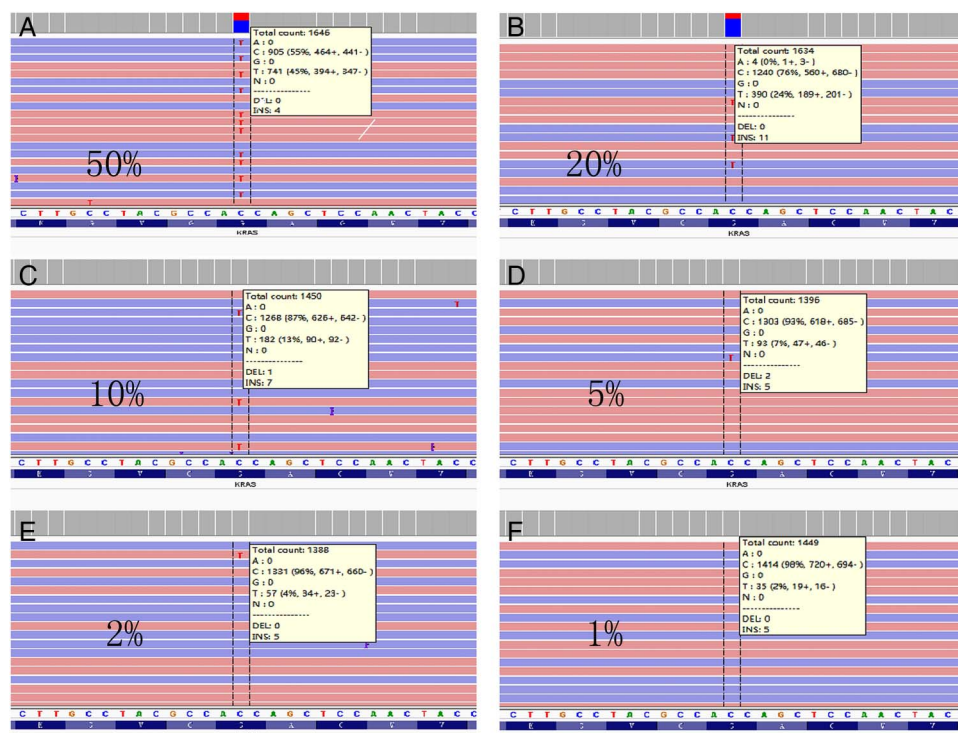| Panc-1in BxPC-3 | KRAS p.G12D (c.35G>A) | | TP53 p.R273H (c.818G>A) | |
	Returned variant frequency (%)	Coverage (X)	Returned variant frequency (%)	Coverage (X)
100% (undiluted)	68.0	1599	100	286
50% (1:1)	45.0	1644	43.6	219
20% (1:4)	24.0	1629	33.3	244
10% (1:9)	12.6	1448	15.4	269
5% (1:19)	6.6	1395	6.4	273
2% (1:49)	4.1	1388	4.5	220
1% (1:99)	2.4	1414	2.2	285

Figure 2 Detection of *KRAS* mutation p.G12D (c.35G>A) in serially diluted DNA isolated from formalin-fixed paraffin-embedded (FFPE) human pancreatic cancer cell lines with known mutation statuses by Ion Torrent PGM (A–F: 50%, 20%, 10%, 5%, 2% and 1% dilutions, respectively).
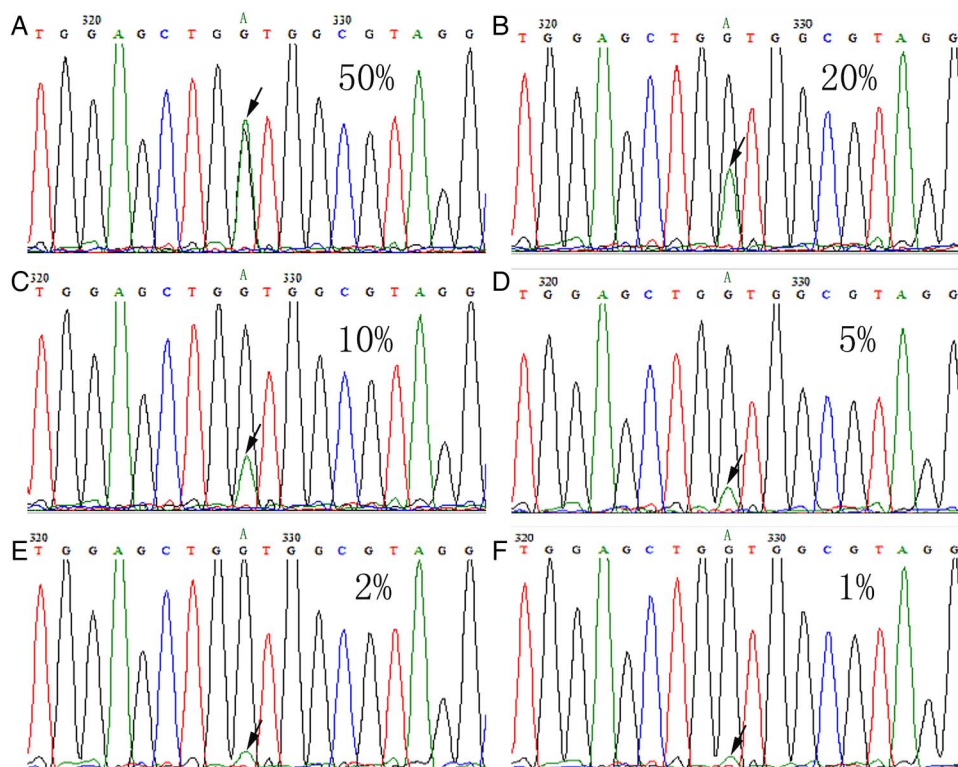

Figure 3 Detection of *KRAS* mutation p.G12D (c.35G>A) in serially diluted DNA isolated from formalin-fixed paraffin-embedded (FFPE) human pancreatic cancer cell lines with known mutation statuses by Sanger sequencing (forward sequencing) (A–F: 50%, 20%, 10%, 5%, 2% and 1% dilutions, respectively).
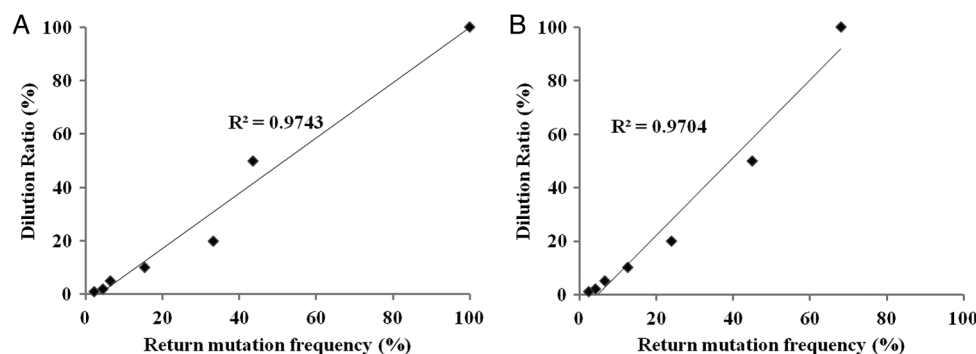
Figure 4 The linearity between the dilutions and calculated mutation frequencies in the dilution series experiment performed using Ion Torrent PGM.

used to test archival FFPE samples for *KRAS* mutations in clinical practice, the feasibility of NGS in this regard has not been well established. Several recent studies have shown high concordance rates between the results obtained with 454 or Illumina NGS technology and standard assays for *KRAS* mutation detection, in fresh-frozen and FFPE tissues.[6 7 17 18] Ion Torrent PGM is a new, semiconductor-based, easy-to-operate benchtop NGS platform. Owing to its relatively low data output and fast turnaround time, Ion Torrent PGM is ideal for smaller, focused sequencing projects (eg, targeted gene panel sequencing) and, thus, it is likely to gain clinical relevance.[10] In this study, we evaluated the performance of the Ion Torrent PGM and a ready-to-use targeted cancer gene panel (Ion AmpliSeq Cancer Hotspot Panel v2) for determining *KRAS* mutation status in 51 FFPE colorectal adenocarcinoma samples. Typical Sanger sensitivity is limited to approximately 15%, and NGS techniques are considered to be more sensitive than Sanger sequencing.[6] In our 51 cases, Ion Torrent PGM analysis showed 100% concordance with Sanger sequencing for *KRAS* exon 2 mutation testing. This finding was likely due to the use of histology-guided macrodissection to enrich for tumour cells, and the lowest variant frequency was 22.2% in our study. To

further examine the sensitivity of Ion Torrent PGM for detecting low-frequency variants, we performed a dilution series experiment using FFPE human pancreatic cancer cell lines with known mutation statuses. Our results indicated that Ion Torrent PGM was able to detect SNVs at various rates with excellent linearity and that it was more sensitive than Sanger sequencing. Although the Therascreen assay is also considered to be more sensitive than Sanger sequencing, it was designed to only detect seven specific somatic mutations in exon 2 of the *KRAS* oncogene and thus might produce false-negative results in *KRAS* exon 2 testing.[9 19] In our study, the results of the Therascreen assay showed 98.04% (50/51) agreement with those of NGS/Sanger sequencing. One case with a c.37G>T mutation, which was not among the seven specific mutations examined, was not detected by the Therascreen assay. Overall, our results showed that Ion Torrent PGM was specific and sensitive for *KRAS* exon 2 mutation detection in FFPE CRC samples compared with Sanger sequencing and the Therascreen assay, and that it is appropriate for use in routine clinical testing. Moreover, Ion Torrent PGM provided variant frequency information that could not be obtained by the traditional methods. Consistent with our results, Ion

Table 5 Application of Ion Torrent PGM for extended *RAS* analysis in formalin-fixed paraffin-embedded (FFPE) colorectal cancer samples

| Patient number | Gene | Exon | Ion Torrent PGM | | | Sanger |
			Variants	Coverage (X)	Frequency (%)	Variants
1	*KRAS*	4	p.K117N(c.351A>T)	990	44.4	p.K117N(c.351A>T)
7	*KRAS*	3	p.Q61H(c.183A>C)	847	34.6	p.Q61H(c.183A>C)
12	*KRAS*	3	p.Q61H(c.183A>T)	2012	45.4	p.Q61H(c.183A>T)
17	*KRAS*	4	p.A146T(c.436G>A)	732	36.7	p.A146T(c.436G>A)
28	*KRAS*	3	p.Q61L(c.182A>T)	1141	38.8	p.Q61L(c.182A>T)
39	*KRAS*	4	p.K117N(c.351A>T)	2420	42.6	p.K117N(c.351A>T)
40	*KRAS*	4	p.K117N(c.351A>T)	462	14.3	p.K117N(c.351A>T)
49	*KRAS*	4	p.A146V(c.437C>T)	1070	66.4	p.A146V(c.437C>T)
50	*KRAS*	4	p.A146T(c.436G>A)	526	58.0	p.A146T(c.436G>A)
47	*NRAS*	2	p.G12C(c.34G>T)	502	41.4	p.G12C(c.34G>T)
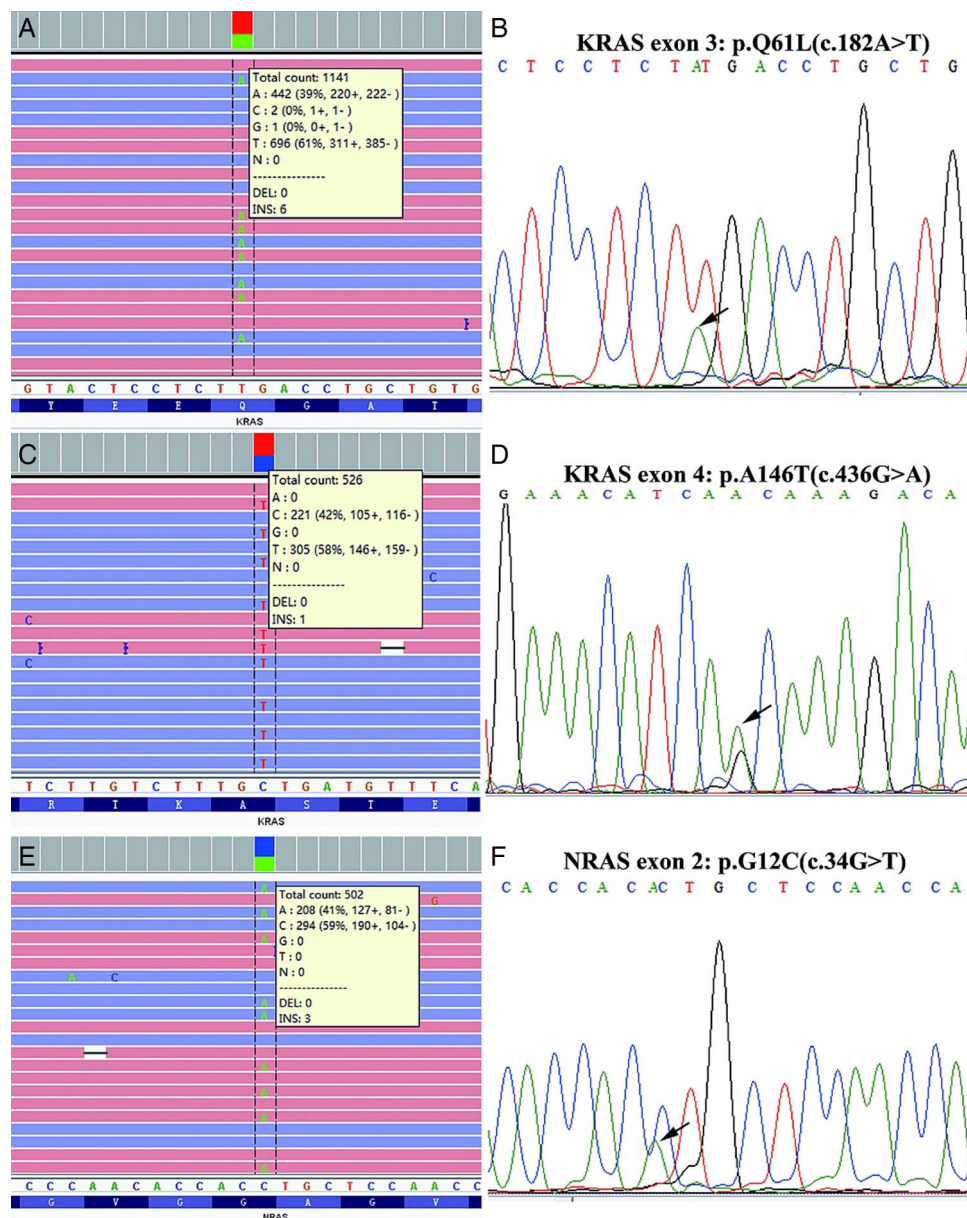
Figure 5 Detection of other *RAS* mutations in case 28 (A and B), case 50 (C and D) and case 47 (E and F), by Ion Torrent PGM and Sanger sequencing (reverse sequencing), respectively.
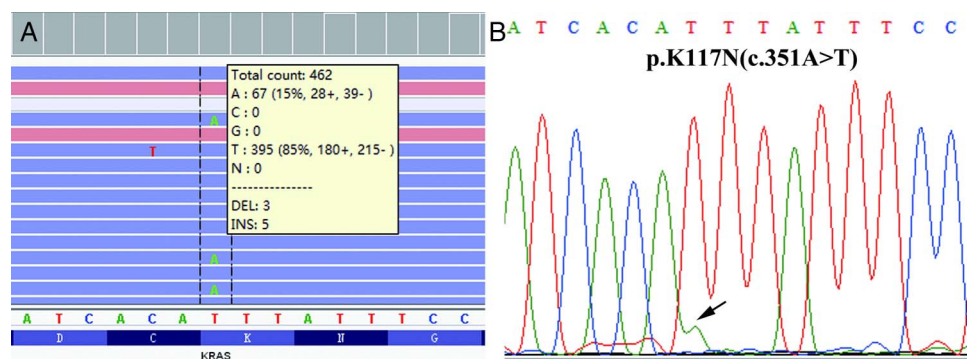
Figure 6 Detection of *KRAS* exon 4 mutation with a relatively low frequency (14.3%) in case 40 by Ion Torrent PGM and Sanger sequencing (reverse sequencing), respectively.

Torrent PGM has been proven to be a reliable mutation screening assay for SNV detection in other tumour types in previous studies.[17 20]

Although *KRAS* exon 2 mutations predict a lack of response to anti-EGFR therapy in patients with metastatic CRC, the absence of a mutation in this gene does not guarantee treatment response to anti-EGFR monoclonal antibody (mAb). Several recent studies have revealed that other *RAS* mutations (in *KRAS* exons 3 and 4 and *NRAS* exons 2, 3 and 4) may also be predictive of anti-EGFR antibody therapy resistance.[4 21] Given this, expanded testing for *RAS* gene mutations, including *KRAS* exon 2 mutations and other *RAS* mutations, has been recommended in the latest NCCN clinical practice guidelines for colon cancer. Ion AmpliSeq Cancer Hotspot Panel v2 was used for Ion Torrent PGM sequencing in our study, and it enabled wide coverage of the *KRAS*, *NRAS* and *HRAS* genes, thereby achieving expansion of *KRAS* exon 2 mutation testing to include *RAS* mutations. Consequently, a considerable proportion of patients with *RAS* mutations were found among our wild-type *KRAS* exon 2 patients (10/38, 26.3%) by Ion Torrent PGM. These patients might also be unlikely to significantly benefit from anti-EGFR mAb therapy. In previous studies, among *RAS* mutations beyond *KRAS* exon 2, mutations in *KRAS* exon 4 were found to occur most commonly in patients with CRC. *KRAS* exon 4 mutations were identified at amino acid residues K117 and A146, and the overall incidence of *KRAS* exon 4 mutations in patients with *KRAS* exon 2 wild-type tumours varied from 3.7% to 9.3%.[4] Given that *KRAS* codon 117 mutation was not evaluated in some studies, the actual incidence of *KRAS* exon 4 mutations might be underestimated.[4 22] We also found exon 4 *KRAS* mutations at K117 and A146 (19 A146T, 1 A146V and 3 K117N) in our 51 cases, at a relatively high frequency (11.8%, 6/51). This should be regarded with caution due to our small sample size, and warrants further exploration in larger studies. Moreover, Ion AmpliSeq Cancer Hotspot Panel v2 was designed to target 'hot spot' regions of 50 oncogenes and tumour suppressor genes (*BRAF, TP53, APC, MLH1, PIK3CA, SMAD4, FBWX7*, etc), and was thus capable of simultaneously detecting other CRC-related gene mutations and providing additional genetic information closely associated with CRC tumourigenesis, prognosis and resistance to therapy. Our results confirmed that Ion Torrent PGM was suitable for expansion of *RAS* mutation testing in FFPE CRC samples and that it has great potential for clinical use.

Importantly, the panel we used for Ion Torrent PGM sequencing included 207 primer pairs contained within a single tube and required as little as 10 ng of FFPE DNA as a template, enabling simultaneous detection of multiple variants of a gene or multiple genes in highly limited specimens, such as biopsy and fine-needle aspirates, facilitating the cost-effective and time-effective sequencing of multiple genes and variants. In contrast, Sanger sequencing and PCR require several micrograms of DNA to detect genetic alterations within a specific region.[15]

Therefore, biopsy specimens may be inadequate for performing multiple genetic tests using these two methods. To make things worse, the need for multiple separate tests causes increases in time and cost. Moreover, Ion Torrent PGM data are automatically analysed and annotated with Torrent Suite Software and Ion Reporter Software, whereas Sanger sequencing data analysis and interpretation require the manual examination of sequencing chromatograms for each gene region and is thus extremely time consuming. As demonstrated in our study, identification of variants that occur at a low frequency in Sanger sequencing chromatograms is especially difficult. Therefore, Ion Torrent PGM is a sample-saving and cost-efficient and time-efficient platform for performing multiplex genetic testing in CRC. Given that few cases in our study had low-frequency mutations, larger study are warranted to further assess the clinical utility of Ion Torrent PGM in detecting low-frequency mutations.

CONCLUSIONS

In summary, we have compared the use of three methods for the detection of *KRAS* mutations in 51 FFPE CRC specimens. Ion Torrent PGM was more specific and sensitive for *KRAS* exon 2 mutation detection than were Sanger sequencing and the Therascreen assay, and it is appropriate for use in routine clinical testing. Moreover, Ion Torrent PGM is a sample-saving and cost-efficient and time-efficient platform for multiplex genetic testing in CRC, and it shows great potential for clinical application to expand testing to include mutations in *RAS* and other CRC-related genes.

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                          ┌─────────────────────────────────────────┐
                          │      Sample collected （n=51）            │
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                          ┌─────────────────────────────────────────┐
                          │  Ion Torrent PGM sequencing （n=51）     │
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RAS mutation:positive （n=23）	RAS mutation: Negative (n=28)

Therascreen KRAS Mutation Kit	Sanger sequencing	Therascreen KRAS Mutation Kit	Sanger sequencing

Positive （n=12）	Negatitive （n=11）	Positive(n=23)	Negatitive(n=0)	Negatitive （n=28）	Negatitive （n=28）
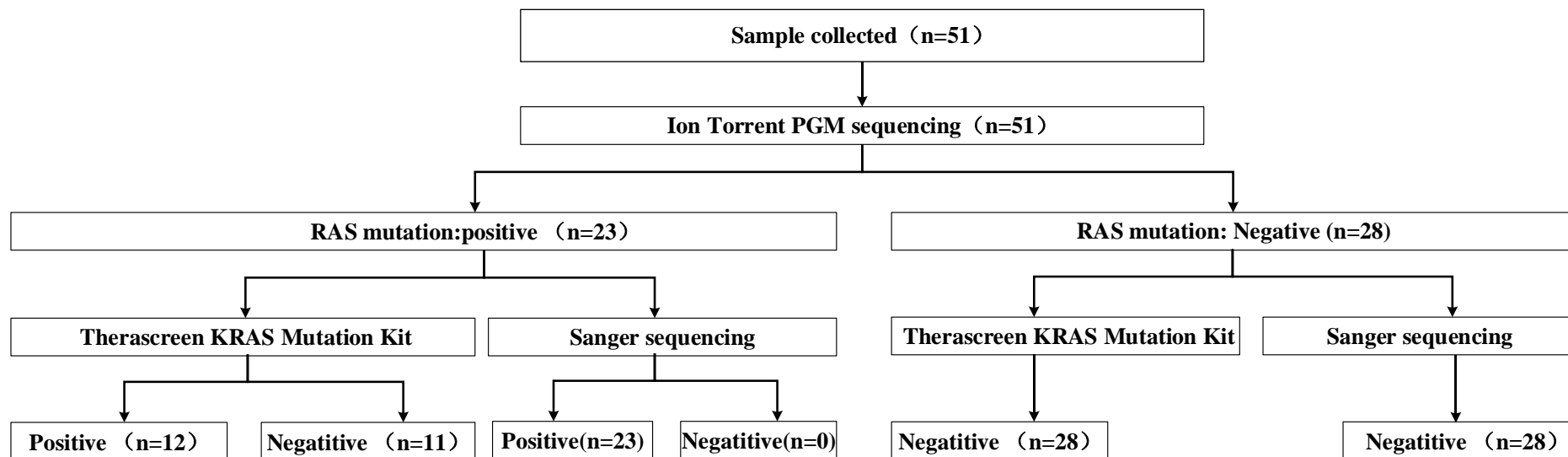
Appendix Figure 1. Detection of mutations in genes other than the *RAS* family in 51 FFPE CRC samples by Ion Torrent PGM using Ion AmpliSeq Cancer Hotspot Panel v2.