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Real-time PCR-based method for the rapid detection of extended *RAS* mutations using bridged nucleic acids in colorectal cancer

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ABSTRACT

Mutations in *RAS* and *BRAF* are predictors of the efficacy of anti-epidermal growth factor receptor (EGFR) therapy in patients with metastatic colorectal cancer (mCRC). Therefore, simple, rapid, cost-effective methods to detect these mutations in the clinical setting are greatly needed. In the present study, we evaluated BNA Real-time PCR Mutation Detection Kit Extended RAS (BNA Real-time PCR), a real-time PCR method that uses bridged nucleic acid clamping technology to rapidly detect mutations in *RAS* exons 2–4 and *BRAF* exon 15. Genomic DNA was extracted from 54 formalin-fixed paraffin-embedded (FFPE) tissue samples obtained from mCRC patients. Among the 54 FFPE samples, BNA Real-time PCR detected 21 *RAS* mutations (38.9%) and 5 *BRAF* mutations (9.3%), and the reference assay (KRAS Mutation Detection Kit and MEBGEN™ RASKET KIT) detected 22 *RAS* mutations (40.7%). The concordance rate of detected *RAS* mutations between the BNA Real-time PCR assay and the reference assays was 98.2% (53/54). The BNA Real-time PCR assay proved to be a more simple, rapid, and cost-effective method for detecting *KRAS* and *RAS* mutations compared with existing assays. These findings suggest that BNA Real-time PCR is a valuable tool for predicting the efficacy of early anti-EGFR therapy in mCRC patients.

1. Introduction

The rat sarcoma (*RAS*) family of oncogenes plays important roles in the RAS-RAF-MEK-ERK-MAPK (RAS-MAPK) signaling pathway, which regulates multiple cellular functions, including cell growth, differentiation, and apoptosis [1]. Mutations in the gene encoding RAS can lead to constitutive activation of the RAS-MAPK pathway by disrupting RAS GTPase activity.

Clinical trials have demonstrated that anti-epidermal growth factor receptor (EGFR) therapies, including cetuximab and panitumumab, are ineffective in metastatic colorectal cancer (mCRC) patients with mutations in *KRAS* exon 2 [2–4]. Recently, several studies reported that mutations in genes encoding components of the RAS-MAPK pathway, including mutations in *KRAS* exons 3 and 4, and *NRAS* exons 2–4, are associated with panitumumab resistance [5–7]. Subsequent studies demonstrated that extended *RAS* (*KRAS* and *NRAS*) mutations have the greatest impact on the efficacy of anti-EGFR therapy in mCRC.

There are several available methods for detecting *RAS* mutations, including direct sequencing, SURVEYOR®-WAVE technology, the BEAMing assay, pyrosequencing, and Luminex®-xMAP® technology [5,8–10]. Direct sequencing is the most frequently-used method for detecting potential gene mutations. However, it requires an enrichment of tumor cells in tissue samples because the detection sensitivity is only 10–25%. All of these methods involve complicated procedures, expensive equipment, and a long turnaround time. Therefore, there is an unmet need for the development of simpler, rapid, cost-effective methods with greater specificity and sensitivity for detecting *RAS* mutations in the clinical setting.

Bridged nucleic acids (BNAs) are a novel type of nucleic acid that exhibit a higher binding affinity to natural nucleic acids (DNA and RNA) and greater nuclease resistance compared with locked nucleic acids and peptide nucleic acids [11]. Previous studies have reported the utility of BNA probes in detecting mutations [12–14]. In this study, we used the BNA Real-time PCR Mutation Detection Kit Extended RAS (BNA Real-time PCR) (RIKEN GENESIS, Tokyo, Japan) to detect extended *RAS* (*KRAS* and *NRAS*) mutations in codons 12, 13, 59, 61, 117, and 146, and *BRAF* mutations in codons 600 and 601 in patients with mCRC. In addition, we analyzed the concordance rate of detected *RAS* mutations between BNA Real-time PCR and the reference assays (KRAS Mutation Detection Kit [Trimgen, MD, USA] or MEBGEN™ RASKET KIT [MBL, Nagoya, Japan]).

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2. Materials and methods

2.1. Samples and DNA extraction

A total of 54 formalin-fixed paraffin-embedded (FFPE) tissue samples from patients with histologically diagnosed mCRC were evaluated. The samples were collected at Fukui Prefectural Hospital (Fukui, Japan) from November 2010 to February 2016. Written informed consent was obtained from all of the patients prior to sample collection or mutation analysis. The FFPE tissue samples were processed by manually microdissecting the tumor area that had been histologically marked by a pathologist. Genomic DNA was extracted using a QIAamp® DNA FFPE Tissue Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. The concentration of genomic DNA was determined using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA).

2.2. BNA Real-time PCR Mutation Detection Kit Extended RAS

BNA Real-time PCR Mutation Detection Kit Extended RAS is a novel, modified version of the real-time PCR method that selectivity amplifies *RAS* (*KRAS* and *NRAS*) mutations in exons 2–4 and *BRAF* mutations in exon 15 using BNA clamping technology (Fig. 1).

Each well of a BNA Real-time PCR reaction mixture comprised master mix, each oligo mix, 500 nM ROX™ reference dye, 0.25 U of BNA Real-time PCR UNG (Uracil-*N*-glycosylase) (RIKEN GENESIS), and 20–100 ng of genomic DNA in a total volume of 25 μl according to the manufacturer's protocol. BNA Real-time PCR was conducted using the StepOnePlus™ Real Time System (Thermo Fisher Scientific) with the following cycling conditions: 50 °C for 3 min (UNG activation), 95 °C for 2 min (UNG deactivation and DNA polymerase activation), and 50 cycles of 95 °C for 30 s and 60 °C for 45 s (collection of FAM™ and VIC® dye signals). The data were analyzed using StepOne™ software v2.2.2 (Thermo Fisher Scientific). The samples were considered positive for a mutation when the threshold line was 0.1 and the threshold cycle (Ct) value of the mutation was < 42.

2.3. Nucleotide sequencing

BNA Real-time PCR products that tested positive for a mutation were sequenced. The products were purified using a QIAquick® PCR Purification Kit (Qiagen) according to the manufacturer's protocol. The sequencing reaction was conducted using the BNA Real-time PCR Extended RAS Mutation Sequencing Primer (RIKEN GENESIS), a BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific), and a 3500Dx Genetic Analyzer (Thermo Fisher Scientific) according to the manufacturers' protocols. The data were analyzed using Sequencing Analysis Software v5.4 (Thermo Fisher Scientific).

2.4. Reference assays

52 FFPE tissue samples were analyzed for the presence of 12 distinct mutations in codons 12 and 13 of *KRAS* exon 2 using a KRAS Mutation Detection Kit according to the manufacturer's protocol at Fukui Prefectural Hospital. If the KRAS Mutation Detection Kit failed to detect a mutation in a given sample, the sample was subsequently analyzed for the presence of 48 distinct mutations in exons 2–4 of *RAS* using a MEBGEN™ RASKET KIT according to the manufacturer's protocol at SRL (Tokyo, Japan) or BML (Tokyo, Japan). Two FFPE tissue samples were analyzed using the MEBGEN™ RASKET KIT only.

2.5. Statistical analysis

The concordance rates between the results of the BNA Real-time PCR assay and the reference assay were statistically analyzed using binomial distribution analysis with a 95% confidence interval (95% CI).

3. Results

3.1. Frequency of RAS and BRAF mutations in CRC samples

Mutations in exons 2–4 of *RAS* and exon 15 of *BRAF* were analyzed in 54 FFPE mCRC samples. *RAS* and *BRAF* mutations identified using BNA Real-time PCR were further characterized using nucleotide sequencing (Fig. 2). BNA Real-time PCR detected mutations in 26 (48.1%) of the 54 samples: 19 (35.2%) mutations in *KRAS* exon 2, 2 (3.7%) other *RAS* mutations (*KRAS* exons 3 and 4, and *NRAS* exons 2–4), and 5 (9.3%) mutations in *BRAF* exon 15. The reference assay detected mutations in 22 (40.7%) of the 54 samples: 20 (37.0%) mutations in *KRAS* exon 2 and 2 (3.7%) other *RAS* mutations. As detected by either one of the methods, 37.0% (20/54) of the samples had a mutation in *KRAS* exon 2, 0% (0/54) had a mutation in *KRAS* exon 3, 1.9% (1/54) had a mutation in *KRAS* exon 4, 1.9% (1/54) had a mutation in *NRAS* exon 2, 0% (0/54) had a mutation in *NRAS* exon 3, 0% (0/54) had a mutation in *NRAS* exon 4, and 9.3% (5/54) had a mutation in *BRAF* exon 15 (Table 1). Among the 29 samples with wild-type *KRAS* exon 2 and *BRAF* exon 15, 2 (6.9%) had other *RAS* mutations.

3.2. The concordance rate of RAS mutation detection between BNA Real-time PCR and the reference assay

The positive, negative, and overall concordance rate between BNA Real-time PCR and the reference assay were 95.5% (21/22) (95% CI: 77.2–99.9%), 100.0% (32/32) (95% CI: 89.1–100.0%), and 98.2% (53/54) (95% CI: 90.1–100.0%), respectively (Table 2). However, the KRAS Mutation Detection Kit detected the *KRAS* p.G13D mutation in a sample that had been determined to be mutation-negative by BNA Real-time PCR. The Ct value of the *KRAS* exon 2 mutation detected by BNA Real-

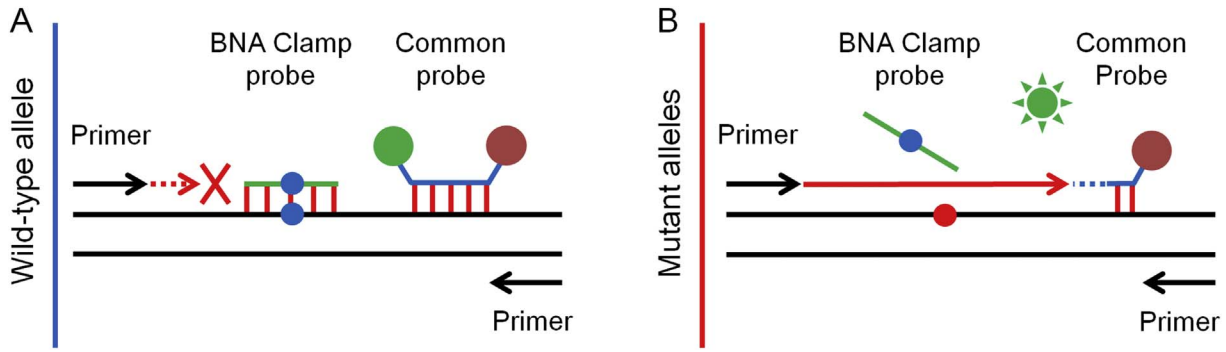

Fig. 1. The BNA Real-time PCR assay. A, The wild-type allele is suppressed by the wild-type allele-specific BNA clamp probe during PCR amplification. B, Mutant alleles are amplified by PCR and fluorescence intensity increases when the probe common to the wild-type and mutant alleles is cleaved. The blue and red circles represent the wild-type and mutant alleles, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

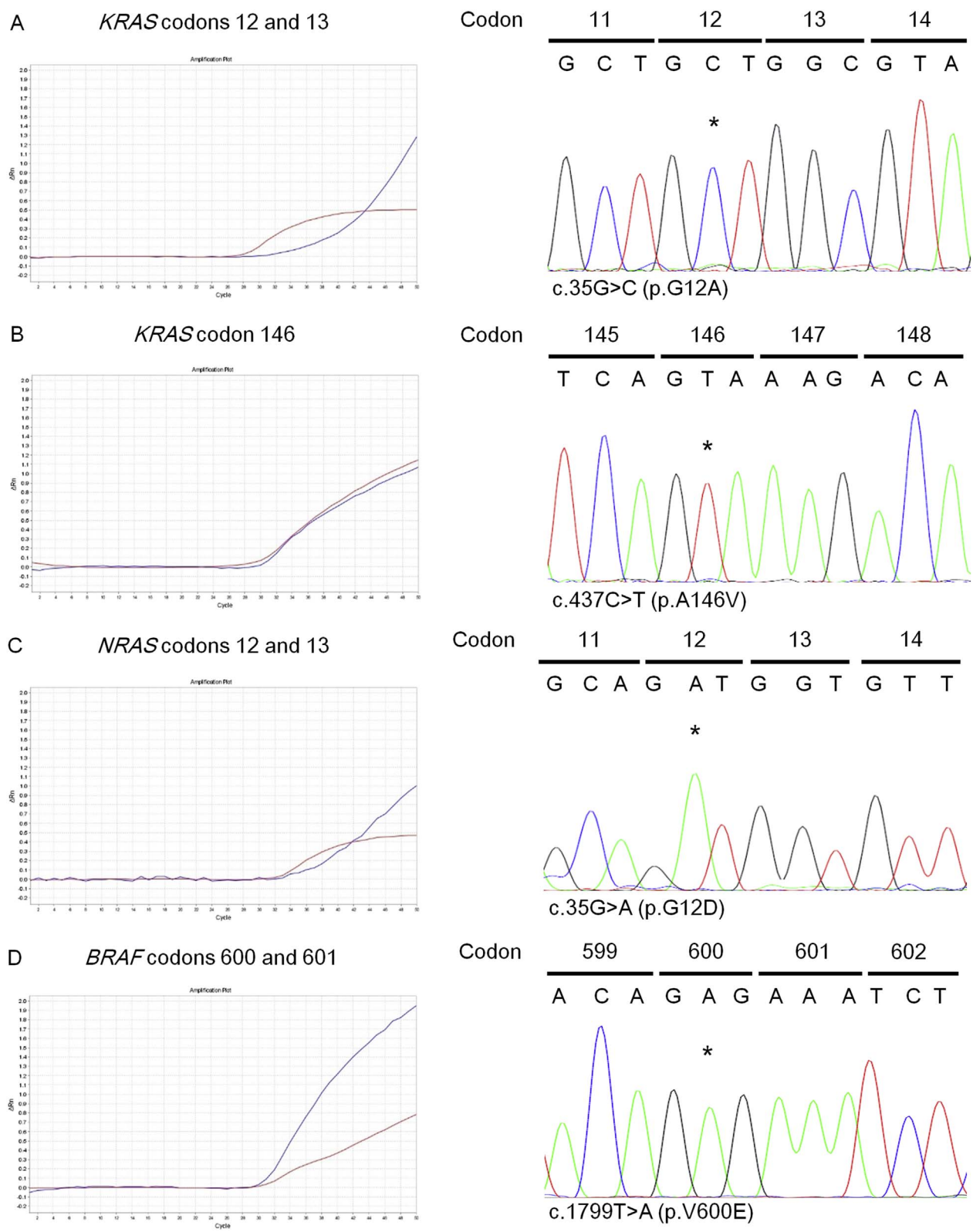

Fig. 2. Representative results of the assays for mutations in *KRAS* codons 12 and 13 (A), *KRAS* codon 146 (B), *NRAS* codons 12 and 13 (C), and *BRAF* codons 600 and 601 (D), Mutations and the internal positive control amplified by BNA Real-time PCR are represented by blue and red lines, respectively. Changes identified by nucleotide sequencing are indicated with an asterisk (*). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Frequency of *RAS* and *BRAF* mutations in metastatic colorectal cancers.

Gene	Exon	Codon	Mutation	No. of cases	%
Wild-type *RAS* and *BRAF*				27	50.0
KRAS	2			20	37.0
		p.G12D	c.35G > A	7	13.0
		p.G12 V	c.35G > T	6	11.1
		p.G12A	c.35G > C	1	1.9
		p.G12S	c.34G > A	1	1.9
		p.G13D	c.38G > A	5	9.3
	3			0	0.0
	4			1	1.9
		p.A146V	c.437C > T	1	1.9
NRAS	2			1	1.9
		p.G12D	c.35G > A	1	1.9
	3			0	0.0
	4			0	0.0
BRAF	15			5	9.3
		p.V600E	c.1799G > A	5	9.3

Table 2
Concordance of *RAS* mutations between the BNA Real-time PCR and the reference assay.

		The reference assay		
		Mutation positive	Mutation negative	Total
The BNA Real-time PCR	Mutation positive	21	0	21
	Mutation negative	1	32	33
	Total	22	32	54
Positive percent agreement	95.5% (21/22) (95%CI: 77.2%–99.9%)			
Negative percent agreement	100.0% (32/32) (95%CI: 89.1%–100.0%)			
Overall percent agreement	98.2% (53/54) (95%CI: 90.1%–100.0%)			

time PCR was > 42.

4. Discussion

The present study demonstrated that BNA Real-time PCR Mutation Detection Kit Extended RAS is a valuable clinical tool for screening *RAS* and *BRAF* mutations in FFPE mCRC samples. The frequency of mutations in *KRAS* exon 2 and *BRAF* exon 15 detected by the BNA Real-time PCR was similar to previous reports [5,6,15,16]. However, the frequency of other *RAS* mutations in the present study was less than what has been reported in previous studies [5–9].

With respect to detecting *RAS* mutations, the positive, negative, and overall concordance rate between the BNA Real-time PCR assay and the reference assay was ≥ 95%. These results indicated that the ability of BNA Real-time PCR to detect *RAS* mutations was comparable to the reference assay. The one discrepancy between BNA Real-time PCR and the KRAS Mutation Detection Kit suggests that the BNA Real-time PCR assay might have a low sensitivity for detecting *KRAS* mutations. The sensitivity of the KRAS Mutation Detection Kit and the MEBGEN™ RASKET KIT to detect mutations in wild-type DNA is 1% and approximately 1–5%, respectively [10,17]. A recent study demonstrated that anti-EGFR therapy was associated with a lower partial response (PR), higher rate of progressive disease (PD), and shorter progression-free survival (PFS) in mCRC patients with even low frequency *KRAS* mutations (< 10%) compared with patients with wild-type *KRAS* [18]. In contrast, a recent study suggested that anti-EGFR therapy was associated with similar overall survival (OS) and PFS rates in patients with very low frequency *KRAS* mutations (< 1%) and patients with wild-type *KRAS* [19]. According to the package insert of the BNA Real-time PCR Mutation Detection Kit Extended RAS, the sensitivity of the assay to detect mutant DNA in a background of wild-type DNA was approximately 1–5% in the dilution test with a control plasmid. A substantial challenge to analyzing genomic DNA from FFPE samples is the spontaneous deamination of cytosine to deoxyuracil, as this leads to a C:G > T:A conversion in nucleotide sequencing. UNG can reduce this effect by specifically removing deoxyuracil [20]. Therefore, UNG was added to the BNA Real-time PCR reaction to prevent the detection of false positive *RAS* and *BRAF* mutations. Therefore, BNA Real-time PCR cannot be used to quantify *RAS* mutations because a standard curve cannot be generated, but it has clinically appropriate sensitivity to detect those.

BNA Real-time PCR is a simple real-time PCR-based method. As the assay does not require expensive equipment or post-PCR procedures, it is a less costly and more rapid (< 2 h) approach than the KRAS Mutation Detection Kit and the MEBGEN™ RASKET KIT [10,17]. Nucleotide sequencing can be used to identify the specific mutation in *RAS* exons 2–4 and *BRAF* exon 15 in BNA Real-time PCR products. In addition, it is easier to confirm the sequencing results of BNA Real-time PCR products compared with conventional PCR products because the BNA clamp probe inhibits amplification of the wild-type allele.

The BNA Real-time PCR assay has two features which can be improved. First, it requires a greater amount of genomic DNA (at least 180 ng/assay) compared with the KRAS Mutation Detection Kit (20–80 ng/assay) and the MEBGEN™ RASKET KIT (50–100 ng/assay) because the BNA Real-time PCR assay requires nine wells/assay and 20–100 ng/well of genomic DNA for detecting *RAS* and *BRAF* mutations according to the package insert. Therefore, for FFPE samples with low levels of genomic DNA, such as those obtained from endoscopic biopsy, a larger sample is needed. The second issue is that the kit only detects *RAS* and *BRAF* mutations, and recent studies indicate that mutations in a several other genes are also associated with resistance to anti-EGFR therapy [6,21]. In the future, it might be necessary to conduct a more comprehensive analysis of mutation status in mCRC patients before initiating anti-EGFR therapies.

In conclusion, BNA Real-time PCR Mutation Detection Kit Extended RAS is a simple, rapid, cost-effective method for detecting *RAS* and *BRAF* mutations, and the ability of the assay to detect *RAS* mutations was comparable to the reference assay. BNA Real-time PCR is a powerful tool for detecting *RAS* and *BRAF* mutations, and predicting the efficacy of anti-EGFR therapies in mCRC patients.

Conflict of interest statement

Some of the reagents for detecting *RAS* and *BRAF* mutations using BNA Real-time PCR were provided by RIKEN GENESIS free of charge. The company did not play a role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

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