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Original

Validation of Pyrosequencing for the Analysis of *KRAS* Mutations in Colorectal Cancer

Yosuke Otake[1,2], Takahiro Fujimori[1], Naohiko Akimoto[1,3], Hiroaki Ikematsu[1,4], Yosuke Okamoto[1,5], Takeshi Yamaguchi[1,6], Kazuhito Ichikawa[1], Shigeki Tomita[1], and Yutaka Saito[2]

[1]*Department of Surgical and Molecular Pathology, Dokkyo Medical University School of Medicine, Tochigi, Japan,* [2]*Endoscopy Division, National Cancer Center Hospital, Tokyo, Japan,* [3]*Division of Gastroenterology, Department of Internal Medicine, Nippon Medical School, Tokyo, Japan,* [4]*Department of Gastorointestinal Oncology and Endoscopy, National Cancer Center Hospital East, Chiba, Japan,* [5]*Division of Gastroenterology and Hepatology, Department of Internal Medicine, Toho University, Omori Medical Center, Tokyo, Japan,* [6]*Department of Surgery 1, Dokkyo Medical University School of Medicine, Tochigi, Japan*

SUMMARY

The use of antibodies against epidermal growth factor receptor (EGFR) in conjunction with conventional chemotherapy for metastatic colorectal cancer (CRC) in patients with *KRAS* wild-type tumors has been proven to be efficacious. Recently, *KRAS* testing prior to anti-EGFR therapy has become mandatory for metastatic CRC patients. Although newly developed pyrosequencing is expected to be one of the high throughput procedures detecting such mutations, the accuracy of the procedure has not been well evaluated. In the present study, we aimed to validate the accuracy, especially the potential for a false-negative result, in detecting *KRAS* mutations by pyrosequencing using cultured tumor cells. DNA extracted from cultured ìNOZî gallbladder cancer cells (known to contain *KRAS* mutation G12V) at concentrations of 1%, 5%, 10%, and 25%, as well as 2 DNA samples extracted from a resected CRC specimen (known to contain another *KRAS* mutation, G12C) at concentrations of 5% and 25%, were prepared. We analyzed *KRAS* mutational status and nonexistent and/or nonfunctional mutations of these 6 samples using pyrosequencing. The *KRAS* mutation detection rates in the 4 NOZ samples (1%, 5%, 10%, and 25%) were 0.37%, 2.79%, 5.28%, and 13.85%, respectively. Some artifacts of *KRAS* mutations unlikely to be present were detected in 1% samples of NOZ at a rate similar to that of the G12V mutation (G12C, 0.29%; G13C, 0.42%). Although the *KRAS* mutation G12C was detected at rates of 1.26% and 6.49% in samples with 5% and 25% DNA extracted from resected CRC specimen, respectively, no other type of *KRAS* mutation was detected in such samples. Pyrosequencing could not detect *KRAS* mutations correctly in the sample containing 1% DNA. This might cause false negatives. A sample mutated DNA concentration of at least 5% was necessary for precise analyses by this procedure.

Key Words: *KRAS* mutation, pyrosequencing, colorectal cancer

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Reprint requests to: Takahiro Fujimori MD, PhD.
Department of Surgical and Molecular Pathology, Dokkyo Medical University School of Medicine, Tochigi, Japan. 880 Kitakobayashi, Mibu, Shimotsuga, Tochigi 321-0293, Japan.

INTRODUCTION

In some clinical trials conducted in the West, antibodies against epidermal growth factor receptor (EGFR), e.g., cetuximab and panitumumab, have been

Table 1 Sample compositions (DNA concentration ; %)

Sample	1	2	3	4	5	6
NOZ[a]	1	5	10	25	—	—
HeLa[b]	99	95	90	75	—	—
Colon cancer cells[c]	—	—	—	—	5	25
Non-tumor cells	—	—	—	—	95	75

[a]NOZ is known to contain the *KRAS* G12V mutation. [b]HeLa is known to contain no *KRAS* mutation.
[c]Colon cancer cells from tissue of resected specimen are known to contain the *KRAS* G12C mutation.

shown to improve the efficacy of conventional chemotherapy regimens and have proven to be efficacious in conjunction with such regimens used in the initial treatment of metastatic colorectal cancer (CRC), especially in patients with *KRAS* wild-type disease[1~4]. However, several studies have indicated that the presence of mutant *KRAS* in metastatic CRC is associated with a lack of response to anti-EGFR antibodies and correlates with a poor prognosis. Mutations in *KRAS* are found in approximately 40% of CRCs, regardless of staging[1,3,5~7]. Circumventing ineffective anti-EGFR therapy in CRC patients with such mutations can avoid unnecessary adverse events, short survival time, and increased medical costs. Prior to treatment with anti-EGFR antibodies in metastatic CRC, *KRAS* testing has become mandatory in the European Union and is recommended in the United States. However, to date, no specific procedure has been recommended to analyze *KRAS* mutations. Direct sequencing and allele-specific polymerase chain reaction (PCR) assay are commonly used for such mutation analysis ; however, direct sequencing requires high tumor cell percentages and good quality material, and sometimes effects an artifact in which some nonspecific mutations are detected due to poor quality of formalin fixation[8,9]. Allele-specific PCR assay is a costly procedure and sometimes derives a false-positive result due to its sensitive detection. Therefore, screening assays that are accurate and efficient at low specimen volume and cost-effective with high throughput are required for the detection of *KRAS* mutations. Pyrosequencing is a real-time quantitative bioluminescence technique for the detection of short stretches of nucleic acid sequences. It is also a promising technology available for *KRAS* mutation screening with reportedly high sensitivity and high throughput. To date, however, false-negative results of pyrosequencing have not been reported when detecting such mutations. The minimum volume of specimen needed to detect a positive result correctly is not known. Therefore, the aim of the present study using cultured tumor cells of the cell line NOZ was to evaluate the volume of specimen necessary for the correct detection of *KRAS* mutations by pyrosequencing.

MATERIALS AND METHODS

Sample preparation

NOZ, a cultured gallbladder cancer cell line known to contain a *KRAS* G12V mutation, and HeLa, a cultured cervical cancer cell line known to contain no *KRAS* mutation, were used for the present study. To determine the minimum DNA concentration for the correct detection of such mutations, DNA extracted from NOZ cells was admixed with DNA from HeLa cells to produce mixtures containing mutant DNA at concentrations of 1% (sample 1), 5% (sample 2), 10% (sample 3), and 25% (sample 4) (Table 1). Furthermore, we prepared surgically resected colon cancer specimens, the cells of which were known to contain the *KRAS* G12C mutation. Then, DNA extracted from the tumor tissue was admixed with DNA from non-tumor cells of the same specimen to produce mixtures containing mutant DNA at concentrations of 5% (sample 5) and 25% (sample 6) (Table 1).

Mutation detection using pyrosequencing

Five hundred nanograms of each sample was prepared and sent to a commissioned company (Roche

Table 2 Mutation detection rate by pyrosequencing

Sample			1		2		3		4		5		6	
Nucleotide number[a]	Mutation type	Variant	Detection rate (%)	Number of reads	Detection rate (%)	Number of reads	Detection rate (%)	Number of reads	Detection rate (%)	Number of reads	Detection rate (%)	Number of reads	Detection rate (%)	Number of reads
34	G>T	G12C	0.29	8,738	0.01	21,280	0	8,499	0.22	17,439	1.26	16,299	6.49	13,474
35	G>T	G12V	0.37	8,811	2.79	21,470	5.28	8,575	13.85	17,586	0.04	16,405	0	13,610
37	G>T	G13C	0.42	8,811	0.01	21,470	0.27	8,575	0	17,591	0	16,405	0	13,610
43	G>T		0.2	8,836	5.46	22,804	0	8,596	0.34	17,604	0	16,492	0	13,611
44	G>C		0	8,836	5.49	22,805	0	8,596	0.02	17,604	0	16,492	0	13,611
46	A>C		1.06	8,872	6.66	22,946	0.88	8,607	0.84	17,872	0.94	16,511	1.01	13,626
47	A>T		0	9,778	6.23	23,123	0	9,465	0	19,139	0	17,885	0	14,631
49	A>T		11.13	9,944	7.1	23,126	9.63	9,489	8.27	19,185	8.36	18,017	7.16	14,655
60	G>A		0.27	9,942	0	23,126	0.43	9,492	0.01	19,192	0	18,017	0.26	14,655
73	C>A		1.53	9,930	0.44	23,121	0.87	9,520	0.76	19,190	0.01	18,021	0.04	14,652
75	G>T		0.52	9,930	0.01	23,121	0.27	9,520	0.29	19,190	0	18,021	0.02	14,653
79	C>A		1.31	9,930	0.39	23,120	1.08	9,520	0.72	19,180	0.04	18,021	0.04	14,652
82	T>C		0.01	9,925	0.02	23,119	0	9,520	0.01	19,178	0.51	18,013	0	14,647
91	G>T		1.15	9,902	0.45	23,111	0.75	9,509	0.69	19,178	0	18,012	0.07	14,640
97	G>T		0.44	9,888	0.27	23,111	0.53	9,507	0.3	19,128	0	18,008	0	14,636
100	C>A		0.81	9,873	0.26	23,095	0.5	9,474	0.4	19,131	0	18,008	0.05	14,626
136	T>C		0	9,835	0	23,021	0.51	9,454	0.01	19,061	0.06	18,006	0.03	14,604

[a]Nucleotide number indicates the number from initial nucleotide of initiating codon.

Diagnostics Japan, Tokyo, Japan) for pyrosequencing. Pyrosequencing analysis of these 6 samples was performed according to the manufacturer's recommendations for the commercially available GS Junior Version 2.5 system (Roche Diagnostics Japan, Tokyo, Japan) with the GS Amplicon Variant Analyzer. Three *KRAS* mutations, including 2 involving codon 12 (G12V and G12C) and 1 involving codon 13 (G13C), were targeted for analysis, in addition to 14 nonexistent and/or nonfunctional point mutations on another codons of *KRAS*. The authors were blinded to the procedure followed by the company, and the company did not obtain information about composition of these samples either. After analysis for mutational status, we received the data from the company and identified the samples.

RESULTS

Pyrosequencing was successfully performed in all 6 samples. *KRAS* mutation analysis using NOZ DNA of varying concentrations (samples 1 to 4) revealed G12V mutation detection rates as 0.37%, 2.79%, 5.28 %, and 13.85% (Table 2). The detection rate of this mutation increased with increasing concentrations. However, theoretically nonexistent G12C and G13C mutations were also detected with levels similar to that of G12V in sample 1 (Table 2). *KRAS* mutation analysis using resected CRC specimens demonstrated that the detection rates of the G12C mutation of the lower concentration sample (5% ; sample 5) and higher concentration sample (25% ; sample 6) were 1.26% and 6.49%, respectively (Table 2). In sample 2, 4 nonexistent and/or nonfunctional point mutations were detected at a rate of more than 5%. One nonexistent point mutation (49 ; A>T) was detected in all samples at a rate of more than 7% (Table 2).

DISCUSSION

This is a fundamental investigation of the detection capability of *KRAS* mutations by pyrosequencing evaluated using DNA of the NOZ cell line in varying concentrations. We concluded that it was impossible to detect specific *KRAS* mutations for identification at a sample mutated DNA concentration of 1% ; therefore, a mutated DNA concentration of at least 5% was necessary to detect *KRAS* mutations correctly. However, a sample mutated DNA concentration of 5% was sufficient to detect such a mutation on the basis of results of the evaluation conducted using resected CRC speci-

mens.

Pyrosequencing is a recently developed and easily available DNA sequencing technique having high analytical sensitivity for mutation detection. It is efficient and flexible in allowing high sample throughput and simultaneous use of different assays during the same run. In principle, the addition of each nucleotide during DNA chain elongation releases pyrophosphate, which through a series of enzymatic reactions is converted to a light signal of intensity proportional to the amount of nucleotide incorporated[10]. Pyrosequencing detects the light signal and decodes the sequence of bases. This allows the identification and quantification of a predetermined nucleotide at a given position within the DNA sequence. In over 10 studies, pyrosequencing was compared for sensitivity with conventional procedures such as DS and allele-specific PCR assay of resected CRC specimens[11~17]. In most of these reports, superior performance of pyrosequencing with higher sensitivity, even with small amounts of sample, was found. To date, however, it has not been reported how much minimum specimen is needed to detect a positive result correctly. Although the results of the evaluation we conducted using NOZ indicated that pyrosequencing could analyze *KRAS* mutations quantitatively, this procedure led to some false detections. In the sample of the lowest concentration (1%) of NOZ DNA, in particular, other types of *KRAS* mutations, which were theoretically nonexistent, were also detected at a detection rate similar to that of the G12V mutation. Therefore, the correct type of *KRAS* mutation could not be identified in such samples. This suggested that a sample with 1% or less mutated DNA concentration could cause false negatives when detecting *KRAS* mutations by pyrosequencing. According to a clinical report describing sample volume, *BRAF* mutational status of endoscopic biopsies and resected specimens of CRC analyzed by pyrosequencing showed a concordance of 99.2%[18].

Rapid progress has been made in developing procedures for mutation analysis. Although more sensitive and low-cost procedures will be developed in the future, they should be evaluated not only for their sensitivity but also for false-negative rates before clinical application. Evaluation for *KRAS* mutational status using cultured tumor cells in varying concentrations is considered useful for determination of the required minimal amount of sample. Furthermore, the credibility of such an analysis will be increased when samples remain anonymous, as in the present study. This fundamental evaluation will be important and helpful when findings of mutation detection differ between analyses using microdissected samples from tumors and samples without microdissection from resected specimens.

CONCLUSIONS

A mutated DNA concentration of at least 5% was necessary to detect *KRAS* mutations correctly by pyrosequencing.

Conflict of interest
The authors declare no conflict of interest.

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