Biochemical and Biophysical Research Communications 395 (2010) 158–162

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The frequency of KRAS mutation detection in human colon carcinoma is influenced by the sensitivity of assay methodology: A comparison between direct sequencing and real-time PCR ☆

Takashi Kobunai [a,b,*], Toshiaki Watanabe [a], Yoko Yamamoto [a], Kiyoshi Eshima [b]

[a] Department of Surgery, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan
[b] Tokushima Research Center, Taiho Pharmaceutical Co. Ltd., 224-2 Ebisuno, Hiraishi, Kawauchi-cho, Tokushima 771-0194, Japan

ARTICLE INFO

Article history:
Received 25 March 2010
Available online 31 March 2010

Keywords:
Colon cancer
Detection method
Frequency
KRAS mutation

ABSTRACT

Purpose: Kirsten rat sarcoma (KRAS) gene mutations occur early in the progression of colorectal adenoma to carcinoma. The mutation status of the KRAS gene determines the benefits of molecular targeting drugs in patients with advanced colorectal cancer, although many methods are available to detect such mutations. The purpose of this study was to evaluate the influence of assay sensitivity on the detection frequency of mutated genes. *Methods:* Colorectal tumors in 224 colorectal cancer patients were characterized for KRAS mutations using PCR amplification following by direct sequencing as well as a peptide nucleic acid (PNA)-clamp real-time PCR-based assay. *Results:* KRAS mutations were observed in 32.1% (72/224) patients by direct sequencing, and 43.3% (97/224) by PNA-clamp PCR. The chi-square test revealed that the difference in the frequency of KRAS mutations determined by direct sequencing and PNA-clamped PCR (threshold for 1% detection) was statistically significant ($p < 0.015$). *Conclusions:* Our data suggest that assay method sensitivity clearly influences the detection frequency of mutated genes. As more sensitive assays detect more mutated genes in clinical samples, this must be taken into consideration when determining KRAS gene status in clinical practice.

1. Introduction

Mutations in the Kirsten rat sarcoma (KRAS) oncogene are frequently found in human cancers, particularly those of the pancreas, gall bladder, bile duct, thyroid gland, non-small cell lung cancer, and colorectal cancer [1–4]. The presence of these mutations may determine the prognosis and drug response to new cancer therapies targeting the K-*ras* protein pathway [5].

Cetuximab and panitumumab, the monoclonal antibodies (mcAbs) used to target the epidermal growth factor receptor (EGFR), were recently approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) for use as single agents or in combination with other chemotherapeutic drugs in the treatment of metastatic colorectal cancer (mCRC). However, the McAbs only benefit a subset of patients that express the wild-type K-*ras* protein; tumors with mutated K-*ras* do not respond to this treatment modality [6–9]. A significant improvement in overall survival and progression-free survival was observed in patients with wild-type K-*ras* tumors following treatment with cetuximab compared with supportive care alone, but not in those with mutated K-*ras* tumors [8]. It is therefore important that the KRAS mutation status be determined precisely to maximize the patient's benefit in a clinical setting.

While a variety of methods are available for the detection of KRAS mutations, nested PCR followed by direct sequencing and allele-specific real-time PCR have been widely utilized so far. We hypothesized that differences in the sensitivity of mutation screening methods may influence mutation detection frequency. To test this, we compared the frequency of KRAS mutations detected in clinical colon cancers by two discrete methods. The first involved classical nucleotide sequencing analysis in which PCR amplification is followed by direct sequencing, and the second is a more sensitive method involving the peptide nucleic acid (PNA)-clamp real-time PCR-based assay [10].

2. Materials and methods

2.1. Cell culture

SW480 and HCT116 cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Cells were

☆ *Financial support:* Ministry of Education, Science, Sports, Culture, and Technology of Japan; the Ministry of Health, Labour, and Welfare; in part by TAIHO Pharmaceutical Co., Ltd.

* Corresponding author at: Department of Surgery, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan. Fax: +81 3 5375 6097/ +81 88 665 6554.
E-mail address: azteca_tk@yahoo.co.jp (T. Kobunai).

0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.03.167

grown in 10-cm culture dishes. SW480 cells were maintained in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum, and HCT116 cells were cultured in McCoy's 5a medium supplemented with 10% fetal bovine serum.

2.2. Patient samples

Two hundred twenty-four patients with surgically resected colorectal cancer were included in this study. Informed consent was obtained from the patients for the collection of tumor specimens, and the study protocol was approved by the local ethics committee. Samples were taken from surgically resected tumors, snap-frozen in liquid nitrogen and stored at −80 °C until required as described previously [11].

2.3. Preparation of genomic DNA

Genomic DNA was extracted from tumor samples using the QIAamp DNA mini kit (Qiagen Inc., Valencia, CA), according to the manufacturer's instructions. Genomic DNA from cell lines was isolated using the Wizard genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer's protocol.

2.4. Nucleotide sequence analysis

Mutational analysis of KRAS was performed using genomic DNA. The primers used to evaluate exon 2 of KRAS were as follows: KRAS forward: 5′-TAAGGCCTGCTGAAAATGACTG-3′, and KRAS reverse: 5′-TGGTCCTGCACCAGTAATATGC-3′. PCR amplification was performed in a total volume of 20 μl, containing 2 μl DNA (10 ng), 10 μl 2× HotStarTaq Plus Master Mix (Qiagen) and 100 nM of each primer. PCR fragments were cleaned with ExoSAP-IT treatment (USB Corporation, Cleveland, OH), sequenced on an ABI 3730 Capillary Genetic Analyzer (Applied Biosystems Inc., Foster City, CA), and analyzed in both sense and antisense directions. DNA sequence analysis was performed using Sequencing Analysis software v5.2 (Applied Biosystems Inc., Foster City, CA), followed by visual analysis of each electropherogram by two independent investigators. The appropriate positive and negative controls were included for the evaluated exon.

2.5. PNA-clamp real-time PCR SYBR assay

KRAS mutation status of DNA, extracted from cancer cell lines and patient samples, was determined using the PNA-clamp real-time PCR SYBR assay. PCR amplification was performed in a total volume of 25 μl, containing 2 μl DNA (20 ng), 12.5 μl 2× Fast Real-Time SYBR Green PCR master mix (Qiagen), 75 nM of each primer and 400 nM PNA (Panagene, Daejeon, Korea). The without PNA-clamp control lacked PNA. PCR cycling conditions were 95 °C for 5 min followed by three-step cycling 40 cycles of 95 °C for 10 s, 70 °C for 10 s and 60 °C for 30 s followed by a melting curve from 60 to 95 °C. In each experiment, PCR reagents without template were run in parallel as no template controls. The PNA clamp was designed to hybridize to the wild-type (wtDNA) KRAS allele surrounding codons 12 and 13. PCR was performed using the forward primer 5′-ATCGTCAAGGCACTCTTGCCTAC-3′, the reverse primer 5′-GTACTGGTGGAGTATTTGATAGTG-3′ and the PNA-clamp H2N-TACGCCACCAG CTCC-CON2H. PNA hybridization securely inhibits annealing of the partially overlapping reverse primer and inhibits amplification of wtDNA at the KRAS allele. The PNA/DNA hybrid is unstable due to base pair mismatch and therefore does not inhibit Taq polymerase from extending the reverse primer on mutated tumor DNA. The threshold cycle (Ct value) was automatically calculated from PCR amplification plots in which fluorescence was plotted against the number of cycles. Delta-Ct values were calculated as the Ct value of PCR with PNA minus the Ct value of PCR without PNA. Thus, higher delta-Ct value mean that PNA effectively inhibits PCR amplification of wtDNA.

2.6. Statistical analysis and software

Categorical data analysis was conducted using the chi-square test with JMP5.0 software (SAS Institute, Cary, NC). Regression analysis was performed with KaleidaGraph software (Synergy software, Reading, PA). All differences were considered statistically significant if the p value was <.05.

3. Results

3.1. Patient characteristics

Table 1 summarizes patient demographic data and tumor characteristics. The average patient age was 63.3 years (range 32–88 years). Most patients (93%) had well or moderately differentiated cancers. The mean tumor length was 4.5 cm.

3.2. Detection of KRAS gene mutations by direct sequencing

In the colorectal cancer cell line, homozygously mutated alleles in SW480 and heterozygously mutated alleles in HCT116 cells were subjected to direct sequence analysis. In the case of the SW480 cell line, a GGT → GTT mutation in codon 12 was observed, while in the HCT116 cell line, a GGC → GAC mutation in codon 13 was found. Serial dilutions of mutant and wild-type alleles were subjected to direct sequence analysis, with at least 20–30% of mutated DNA required to assess their status (data not shown). Next, codons 12 and 13 of the KRAS gene were studied in 224 patients. Of these, 72 (32.1%) had KRAS mutations (Table 2). This mutation frequency is not statistically significant when compared to the COSMIC database (chi-square test, $p = 0.78$) [12]. The most frequently observed gene mutations in codon 12 are G → A transitions, then G → T and G → C transversions, accounting for 51%, 41% and 8%, respectively. Of the total mutations, 71% and 29% were observed in codons 12 (GGT) and 13 (GGC), respectively.

3.3. Validation of PNA-clamp real-time PCR techniques

Real-time PCR was performed and the amplification plot is shown in Fig. 1A. The PCR reactions when genomic DNA (gDNA) from SW480 was used as the template, and the amplification plots either with or without PNA were almost identical. However, in the case of gDNA from normal colon tissues carrying the wild-type

Table 1
Patient characteristics.

Characteristic	*n*
Age (years), mean (sd)	63.3 (12.2), range (32–88)
Gender (male/female)	141/83
T classification	
T1, 2	56
T2	109
T3	59
Size (mm), mean (sd)	45.2 (23.6), range 10–150
Site	
Colon	17
Rectum	107
Histological type	
Well-differentiated	149
Moderately differentiated	60
Poorly differentiated	7
Others	8

Table 2
Comparison of KRAS mutations in codons 12 and 13.

Mutation	Present study	COSMIC database[b]
G12D (GGT → GAT)	24	1864
G12V (GGT → GTT)	17	1172
G12C (GGT → TGT)	4	440
G12A (GGT → CGT)	2	316
G12R (GGT → GCT)	2	60
G12S (GGT → AGT)	2	369
G13D (GGC → GAC)	21	811
G13C (GGC → TGC)	—	28
Others	—	50
Total patients with mutation (%)[a]	72 (32.1)	5111 (31.3%)
Total patients	224	16,345

[a] Statistically insignificant ($p = 0.78$, chi-square test).
[b] Data base search performed February, 2009.

KRAS allele, the presence of PNA caused a delay in PCR amplification. To assess the detection ability of the PNA-clamp real-time SYBR assay, gDNA from SW480 cells was serially diluted with wild-type DNA from normal colon tissues in the following concentrations: 100%, 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0.78%, 0.39%, 0.19%, and 0.098%. The PNA-clamp PCR assay was carried out using 2 ng template DNA from each of the prepared mixtures. The amplification plot and a linear relationship between delta-Ct values and mutant DNA concentration showed that KRAS mutations can be specifically detected at 1024-fold dilution (Fig. 1B and C). Regression analysis of the mutant dilution logarithmic value and delta-Ct values enabled the estimation of threshold delta-Ct values assumed to be a fraction (percent) of mutant DNA in the test sample.

3.4. Detection of KRAS gene mutations by PNA-clamp real-time PCR

The delta-Ct values for 224 clinical colon cancer samples determined by real-time PCR are shown in Fig. 2. Values for SW480 and KRAS wild-type DNA from normal colon tissue are −1.8 and 6.8, respectively. Visual inspection of Fig. 2B clearly reveals the

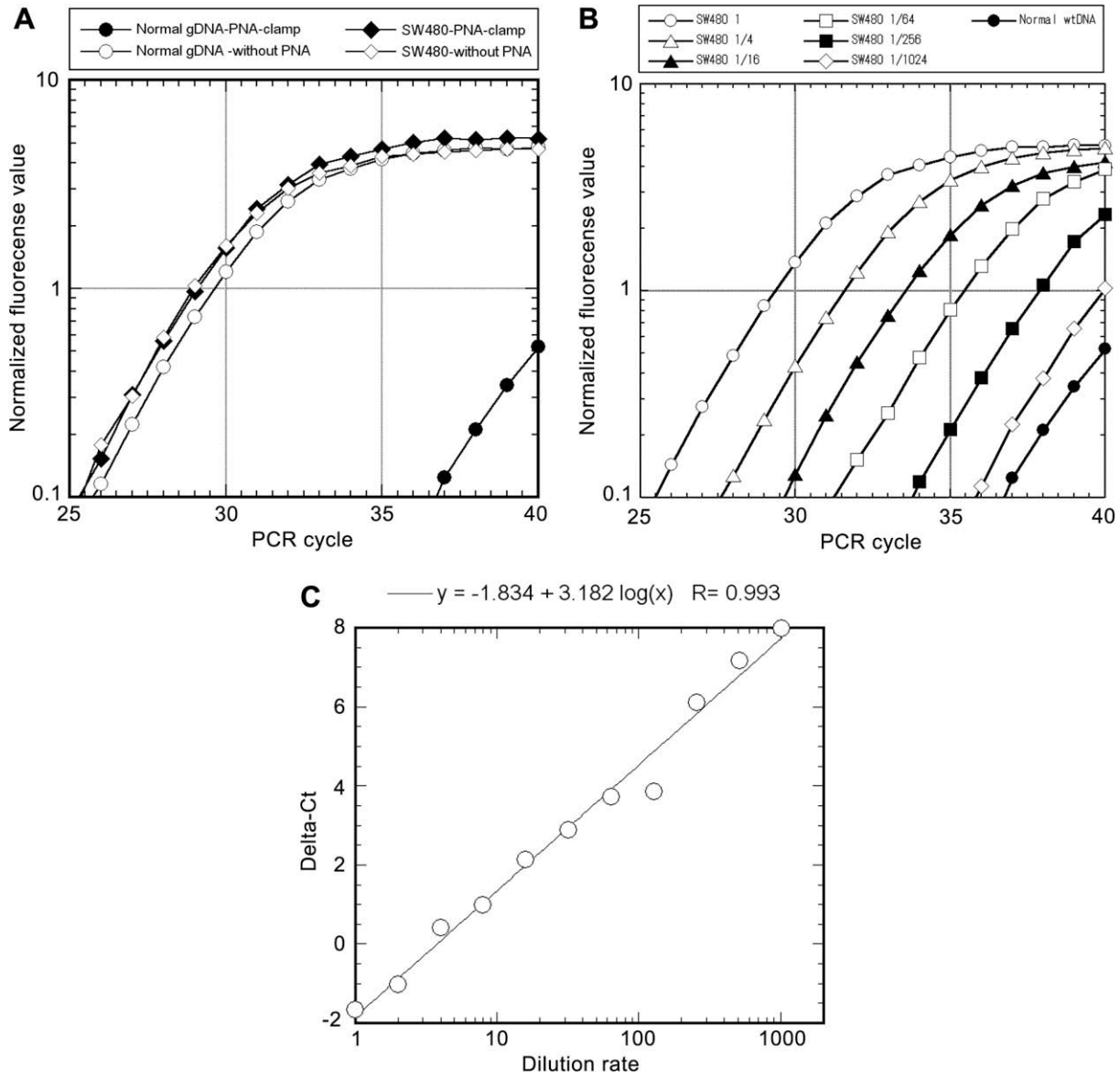

Fig. 1. Amplification plot of real-time SYBR PCR with or without PNA. (A) Normalized fluorescence value of reporter dye (SYBR) divided by fluorescence of passive reference dye (ROX) minus baseline (*y*-axis) plotted against PCR cycles (*x*-axis). (B) Amplification plot of PNA-clamp SYBR PCR for dilutions of DNA from SW480 cells containing KRAS mutation in wild-type DNA from normal colon tissues. PNA-clamp SYBR PCR detected KRAS mutant DNA at 1024-fold dilution. (C) Delta-Ct values plotted against dilution rate, inverse value of the concentrations of mutated DNA in wild-type DNA multiplied by 100.

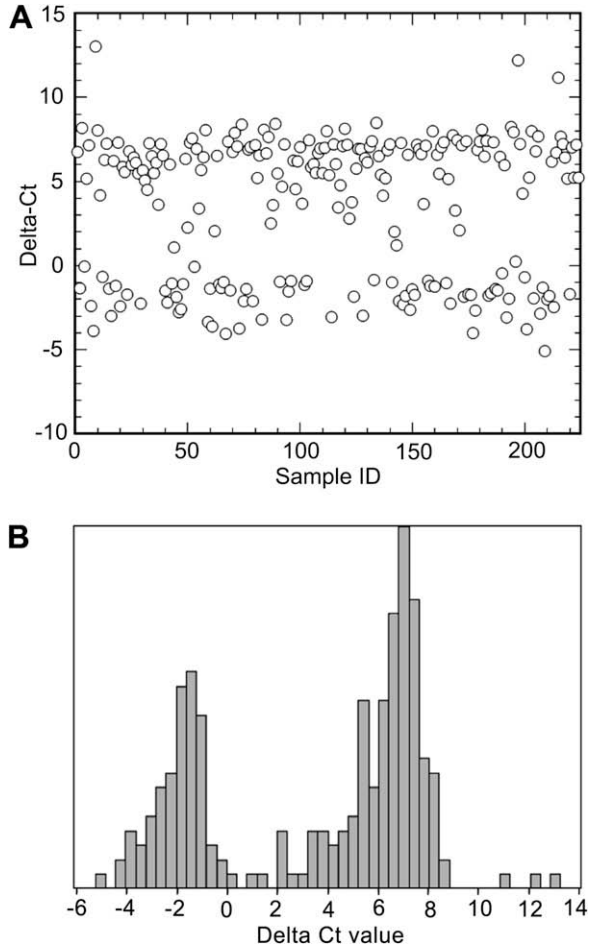

Fig. 2. Delta-Ct values of 224 clinical colon cancer samples. (A) Delta-Ct values plotted against sample ID. (B) Histogram of delta-Ct values.

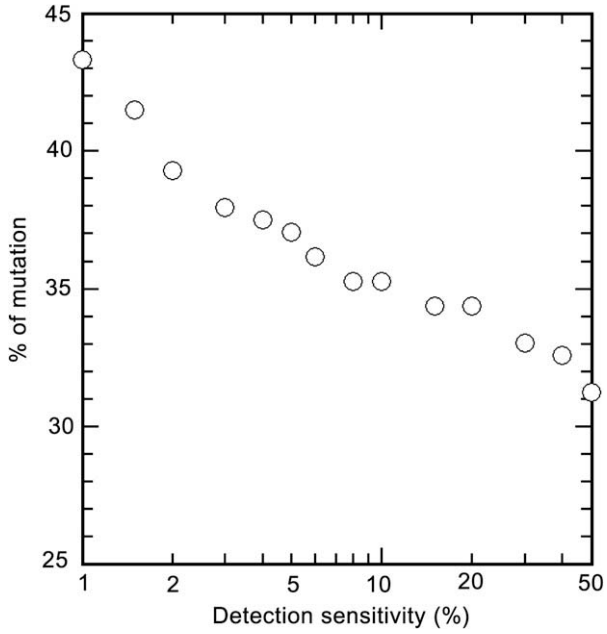

Fig. 3. Relationship between detection sensitivity of PNA-clamp SYBR assay and estimated frequency of KRAS mutation in 224 colon cancer tested.

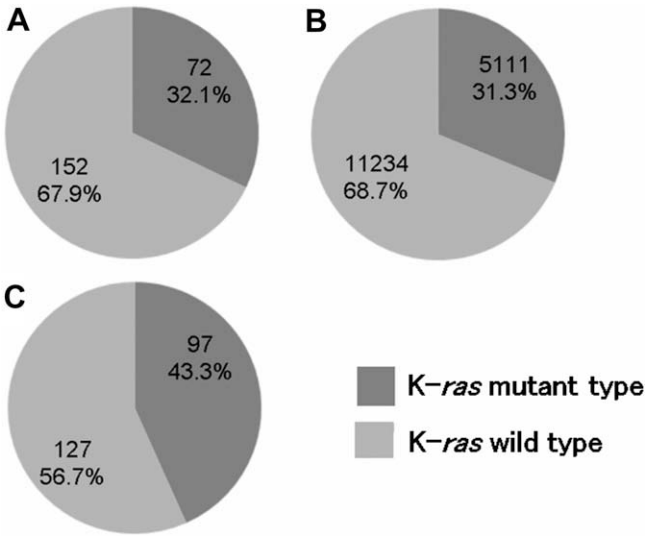

Fig. 4. Pie chart of KRAS mutation frequency detection. (A) Mutation frequency determined by direct sequencing. (B) KRAS mutation frequency in codon 12 and 13 curated in COSMIC database (SANGER Institute). (C) Mutation frequency determined by PNA-clamp real-time PCR.

presence of two peaks around −1.7 and 6.7, indicating that the delta-Ct values derived from clinical samples are symmetrically distributed around the two data points, namely mutated and wild-type KRAS.

The results presented in Fig. 3 depict a relationship between detection sensitivity of the PNA-clamp SYBR assay and the estimated frequency of KRAS mutations. When the cut off value of 4.53 was used for the PNA-clamp SYBR assay to detect 1% mutant DNA, the frequency of KRAS mutations was estimated to be 43.3%. The chi-square test revealed that the difference in the frequency of KRAS mutations determined by direct sequencing and PNA-clamped PCR (threshold for 1% detection) was statistically significant ($p < 0.015$) (Fig. 4).

4. Discussion

In the present study, we demonstrate that differences in the sensitivity of mutation screening methods clearly influence the frequency of mutation detection in colon cancer patients. While direct sequencing detected 72 KRAS mutations in codons 12 or 13 from 224 patients (frequency, 32.1%), PNA-clamp real-time PCR detected a statistically significant higher frequency of mutation, at 43.3%.

The COSMIC (Catalogue of Somatic Mutations in Cancer) public mutation database is the most comprehensive database storing somatic mutation data in a single location [12], storing over 65,000 mutations from 290,000 tumor samples. Mutation data and associated information were extracted from primary literature and entered into the COSMIC database. From 368 papers reviewed between 1992 and 2008, 5111 KRAS mutations in codons 12 or 13 have been extracted from over 16,345 colon cancer samples. This mutation frequency of 31.3% closely approximates the frequency detected by the direct sequencing method in the present study.

On the other hand, recent clinical data found the KRAS mutation frequency to be over 40% in colorectal cancer. A panitumumab clinical phase III study reported by Amado et al. identified KRAS mutations in 184 (43.1%) of 427 patients with mCRC using allele-specific real-time PCR [13]. A similar frequency was also reported by Bokemeyer et al. in a randomized phase II OPUS trial, where KRAS mutations were detected in 99 (42.5%) of 233 patients with mCRC using a sensitive quantitative PCR-based assay [14], while Karapetis et al. reported KRAS mutations in 41.6% patients in a NCIC CTG CO.17

phase III study [8]. Recent clinical studies often utilize a highly sensitive method for KRAS mutation analysis, such as real-time PCR which can detect just 1% of mutant DNA in a small sample. Thus, the reported frequencies in these reports are higher than those in the COSMIC database and more closely resemble those of our own study as determined by the PNA-clamp SYBR assay.

Early studies used allele-specific radio-labeled oligonucleotides to detect specific mutations. In addition, single strand conformational polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), heteroduplex analysis and DNA microarray technology have all been widely used for the detection of somatic mutations. Pyrosequencing technology is, by contrast, a relatively new technique that uses luciferase–luciferin luminescence as a signal for nucleotide incorporation into a PCR template DNA [15]. However, nucleotide sequencing that identifies the presence of any mutation in a complete gene is still a gold standard method. There are literatures which suggest methodology may influence the frequency at which mutations are detected [16–18], although conflicting results have been reported that there was no statistical difference between the mutation rate detected by sequencing and allele-specific methods in 2572 patients participated to the multicenter "RASCAL" study [19].

The sensitivity of each assay also depends on the quality of the sample examined. We used fresh frozen samples as a source of template DNA for both sequencing and PCR. However, many clinical studies relied on formalin fixed paraffin-embedded samples [8,13,14]. In such cases, the DNA might be chemically modified by fixation, so that the reaction efficiency is influenced by fixation conditions such as the concentration of formalin and fixation temperature [20].

In a clinical setting, the identification of KRAS mutations should be performed by a well controlled method, as just a small difference in the mutation frequency due to assay sensitivity would directly affect colon cancer patients because of an inappropriate choice of treatment strategy. Moreover, the development and administration of new drugs may become relevant to the sensitivity of mutation detection methods.

There are some limitations to the present study. The first is a lack of clinical outcomes of EGFR target therapy. In Japan, phase III clinical trials for EGFR targeting drugs such as cetuximab and panitumumab have not yet started for colorectal patients. If the KRAS mutation status can be recalculated, it might be clear whether the 10% of patients with mutated KRAS, who might have been designated KRAS wild type by direct sequencing, could receive clinical benefits or not. The second point is the assay sensitivity of the PNA-enriched PCR method. We detected mutant DNA diluted over 100-fold with wild-type DNA. Recent studies reported that when high-fidelity DNA polymerase was used for the PNA-clamp PCR assay, sensitivity increased by more than 10-fold compared with a Taq polymerase assay. Therefore, high-fidelity DNA polymerase may be useful for future studies to reduce the frequency of polymerase-induced errors. However, the detection limit of the present study is comparable with clinically available assay methods, so that the conclusions of this study are useful for understanding current clinical situations.

Considering recent trends in the field of drug development and efforts made by the FDA in providing safe and effective treatment (The Critical Path Initiative: http://www.fda.gov/oc/initiatives/criticalpath/), the introduction of specific tests such as detecting KRAS mutations meets well with public expectations. Determination of the mutation status in a particular gene is becoming a more important issue in the case of personalized medicine, therefore, it must be carefully controlled to prevent the unintentional depravation of therapeutic benefits for some patient populations.

5. Conflicts of interest

None.

References

[1] C. Almoguera, D. Shibata, K. Forrester, et al., Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes, Cell 53 (1988) 549–554.
[2] G.C. Burmer, L.A. Loeb, Mutations in the KRAS2 oncogene during progressive stages of human colon carcinoma, Proc. Natl. Acad. Sci. USA 86 (1989) 2403–2407.
[3] R.A. Hilger, M.E. Scheulen, D. Strumberg, The Ras-Raf-MEK-ERK pathway in the treatment of cancer, Onkologie 25 (2002) 511–518.
[4] I.Y. Tam, L.P. Chung, W.S. Suen, et al., Distinct epidermal growth factor receptor and KRAS mutation patterns in non-small cell lung cancer patients with different tobacco exposure and clinicopathologic features, Clin. Cancer Res. 12 (2006) 1647–1653.
[5] P. Bachireddy, P.K. Bendapudi, D.W. Felsher, Getting at MYC through RAS, Clin. Cancer Res. 11 (2005) 4278–4281.
[6] D.J. Freeman, T. Juan, M. Reiner, et al., Association of K-ras mutational status and clinical outcomes in patients with metastatic colorectal cancer receiving panitumumab alone, Clin. Colorectal Cancer 7 (2008) 184–190.
[7] J.R. Hecht, E. Mitchellx, T. Chidiac, et al., and panitumumab compared with chemotherapy and bevacizumab alone for metastatic colorectal cancer, J. Clin. Oncol. 27 (2009) 672–680.
[8] C.S. Karapetis, S. Khambata-Ford, D.J. Jonker, et al., K-ras mutations and benefit from cetuximab in advanced colorectal cancer, N. Engl. J. Med. 359 (2008) 1757–1765.
[9] J. Tabernero, E. Van Cutsem, E. Diaz-Rubio, et al., Phase II trial of cetuximab in combination with fluorouracil, leucovorin, and oxaliplatin in the first-line treatment of metastatic colorectal cancer, J. Clin. Oncol. 25 (2007) 5225–5232.
[10] B. Taback, A.J. Bilchik, S. Saha, et al., Peptide nucleic acid clamp PCR: a novel K-ras mutation detection assay for colorectal cancer micrometastases in lymph nodes, Int. J. Cancer 111 (2004) 409–414.
[11] T. Watanabe, T. Kobunai, E. Sakamoto, et al., Gene expression signature for recurrence in stage III colorectal cancers, Cancer 115 (2009) 283–292.
[12] S. Bamford, E. Dawson, S. Forbes, et al., The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website, Br. J. Cancer 91 (2004) 355–358.
[13] R.G. Amado, M. Wolf, M. Peeters, et al., Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer, J. Clin. Oncol. 26 (2008) 1626–1634.
[14] C. Bokemeyer, I. Bondarenko, A. Makhson, et al., and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer, J. Clin. Oncol. 27 (2009) 663–671.
[15] A. Poehlmann, D. Kuester, F. Meyer, et al., K-ras mutation detection in colorectal cancer using the pyrosequencing technique, Pathol. Res. Pract. 203 (2007) 489–497.
[16] S.N. Andersen, T. Lovig, J. Breivik, et al., K-ras mutations and prognosis in large-bowel carcinomas, Scand. J. Gastroenterol. 32 (1997) 62–69.
[17] H.J. Andreyev, J.V. Tilsed, D. Cunningham, et al., K-ras mutations in patients with early colorectal cancers, Gut 41 (1997) 323–329.
[18] J. Breivik, G.I. Meling, A. Spurkland, et al., K-ras mutation in colorectal cancer: relations to patient age, sex and tumour location, Br. J. Cancer 69 (1994) 367–371.
[19] H.J. Andreyev, A.R. Norman, D. Cunningham, et al., Kirsten ras mutations in patients with colorectal cancer: the multicenter "RASCAL" study, J. Natl. Cancer Inst. 90 (1998) 675–684.
[20] C.E. Greer, S.L. Peterson, N.B. Kiviat, M.M. Manos, PCR amplification from paraffin-embedded tissues. Effects of fixative and fixation time, Am. J. Clin. Pathol. 95 (1991) 117–124.