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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 ☆

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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

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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