

Potential clinical significance of plasma-based KRAS mutation analysis using the COLD-PCR/TaqMan[®]-MGB probe genotyping method

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Abstract. Despite the improved ability to detect mutations in recent years, tissue specimens cannot always be procured in a clinical setting, particularly from patients with recurrence of tumors or metastasis. Therefore, the aim of this study was to investigate whether plasma is able to be used for mutation analysis instead of tissue specimens. We collected plasma from 62 patients with colorectal cancer (CRC) prior to treatment. DNA extracted from plasma and matched tumor tissues were obtained. Mutations in KRAS were amplified from the tissue specimens and sequenced by regular polymerase chain reaction (PCR) and co-amplification at lower denaturation temperature (COLD)-PCR. Plasma KRAS gene mutation on codon 12 (GGT>GAT) was detected using a nested COLD-PCR/TaqMan[®]-MGB probe. Mutations in plasma and matched tumors were compared. KRAS mutation on codon 12 (GGT>GAT) was found in 13 (21.0%) plasma specimens and 12 (19.4%) matched tumor tissues. The consistency of KRAS mutations between plasma and tumors was 75% (9/12), which indicated a high correlation between the mutations detected in plasma DNA and the mutations detected in the corresponding tumor DNA ($P<0.001$; correlation index, $k=0.649$). Notably, four (6.5%) patients with plasma DNA mutations had no detectable KRAS mutations in the corresponding primary tumors, and three (4.8%) patients with tumor DNA mutations had no detectable KRAS mutations in the corresponding plasma DNA samples. Thus, KRAS mutations in plasma DNA correlate with the mutation status in matched tumor tissues of patients with CRC. Our study

provides evidence to suggest that plasma DNA may be used as a potential medium for KRAS mutation analysis in CRC using the COLD-PCR/TaqMan-MGB probe method.

Introduction

Cancer biomarkers play multiple roles in oncology. They can have prognostic functions by providing information on outcome and patient tractability. Somatic mutations in KRAS are considered a predictive marker of response to therapy in colorectal cancer (CRC), due to their association with clinical resistance to cetuximab and panitumumab, chimeric monoclonal antibodies acting as epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) (1,2). The analysis of KRAS mutations has been approved by the US Food and Drug Administration and European Medicines Agency as a diagnostic tool with which to select colorectal cancer patients eligible to be treated with EGFR-TKIs (1,3).

Although KRAS mutations are detectable in freshly frozen specimens and formalin-fixed paraffin-embedded (FFPE) specimens, some specimens cannot be accessed in a clinical setting, particularly for patients who have had recurrence of tumor and metastasis. However, shed tumor DNA circulating in patient plasma may provide a viable alternative for the identification of tumor mutations and other alterations (4). As a large quantity of wild-type background DNA circulates in the plasma, mutations cannot be sequenced using conventional methods.

Co-amplification at lower denaturation temperature-polymerase chain reaction (COLD-PCR), a novel form of PCR that amplifies minority alleles selectively from mixtures of wild-type and mutation-containing sequences, provides a general platform to improve the sensitivity of DNA variation detection technologies (5,6). DNA genotyping with mutation-specific TaqMan[®] probes (Applied Biosystems) is broadly used in the detection of single-nucleotide polymorphisms but is less frequently used for somatic mutations due to its limited selectivity for low-level mutations (7). Minor-groove binder-based TaqMan probes (TaqMan-MGB) have a higher specificity than common TaqMan probes. We investigated whether combining COLD-PCR with the TaqMan-MGB probe genotyping method

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is able to determine KRAS mutations in plasma from patients with CRC, and we ascertained whether plasma can be used for mutation analysis instead of tissue specimens.

Materials and methods

Plasma sample collection and DNA extraction. We collected plasma from 62 patients with CRC prior to treatment between the years 2009 and 2010. This was carried out as follows. Whole blood specimens were collected in ethylene diaminetetraacetic acid tubes and centrifuged at 3000 rpm for 10 min. Plasma was stored at -80°C until DNA extraction. DNA was extracted from 0.5 to 1.0 ml of plasma using the QIAamp DNA Blood mini kit (Qiagen) following the manufacturer's instructions. Estimated final DNA concentrations generally ranged from 1 to 8 ng/ μ l, with an average of 2.2 ng/ μ l.

Tissue specimen collection and DNA extraction. CRC tissue specimens (n=62) paired with plasma were obtained surgically, and were confirmed by the Department of Pathology. Tissue was stored at -80°C prior to use. DNA was extracted by phenol-chloroform, as follows. We added lysate and protease K, and then placed the specimens overnight in a 60°C waterbath. DNA was extracted by phenol/chloroform/isopentanol the following day. Following salt washing, the sediment was dissolved in quantity-sufficient Tris-EDTA buffer and then preserved at -20°C for use (8).

Determination of the critical denaturation temperature (T_c) for COLD-PCR. We determined T_c experimentally for each amplicon as previously described. In order to determine T_c , the melting temperature (T_m) of the amplicon was first identified. A real-time PCR of the target amplicon was performed in a PCR machine in the presence of 0.1X LCGreen Plus dye using conventional thermocycling conditions, followed by a melting curve analysis (9). A set of COLD-PCR reactions were then carried out at graded temperatures below the T_m from 79 to 82°C to identify the optimal critical denaturation temperature. We found that 81.0°C was the optimal T_c for the fast COLD/direct sequencing, and 81.0 and 80°C for the first and second round COLD-PCR/TaqMan-MGB probe.

Regular PCR and COLD-PCR amplification and direct sequencing for KRAS. Mutations in KRAS on codon 12 (GGT>GAT) were amplified by regular PCR and COLD-PCR following extraction of DNA from the tissue specimens, using the following forward and reverse primers: KRAS-F, 5'-AAG GCC TGC TGA AAA TGA CTG-3' and KRAS-R 5'-GGT CCT GCA CCA GTA ATA TGC A-3' (10). PCR reaction was performed using 25 μ l total volume consisting of 20 ng genomic DNA, a final concentration of 1X PCR buffer (10 mmol/l Tris-HCl, pH 8.3; 50 mmol/l KCl), 2.5 mmol/l MgCl solution, 0.2 mmol/l each dNTP, 0.5 μ mol/l each primer and 1.25 U FastStart Taq Gold DNA polymerase. The regular PCR cycling conditions were as follows: 95°C for 3 min followed by 35 cycles of 95°C for 30 sec, 58°C for 20 sec, 72°C for 20 sec, and a final extension of 72°C for 8 min. The COLD-PCR cycling conditions were as follows: 95°C for 3 min followed by 10 cycles of 95°C for 15 sec, 58°C for 20 sec, 72°C for 20 sec, 40 cycles of 81°C for 1 sec, 58°C for 20 sec, 72°C for

20 sec and a final extension of 72°C for 8 min. The reactions were performed in a Bio-Rad PCR instrument. The size of the product was 166 bp. The PCR products were subjected to sequence analysis.

A nested COLD-PCR/TaqMan-MGB probe to detect KRAS mutations (GGT>GAT) in plasma and tumors. KRAS gene mutation (GGT>GAT) was detected in the plasma and tumor using nested COLD-PCR for 62 CRC patients. The first fast COLD-PCR amplification was performed using the same reaction system as the sequencing. The reaction conditions were as follows: predegeneration at 95°C for 3 min, 10 cycles of degeneration at 95°C for 15 sec, renaturation at 58°C for 20 sec and an extension at 72°C for 20 sec, 40 cycles of degeneration at 81°C for 1 sec, renaturation at 58°C for 20 sec and extension at 72°C for 20 sec and a final extension of 5 min at 72°C in a Bio-Rad PCR instrument. The PCR products were diluted 1000-fold as a template for the second amplification. The second fast COLD-PCR reactions were performed in the presence of 0.2 μ mol/l of a TaqMan-MGB probe (5'-FAM-TTGGAGCTGATGGC-BHQ-MGB-3') that fully matched the mutation-containing sequence. The final concentrations of the other reagents were as follows: 1X PCR buffer (10 mmol/l Tris-HCl, pH 8.3; 50 mmol/l KCl), 2.0 mmol/l MgCl solution, 0.2 mmol/l each dNTP, 0.5 μ mol/l each primer (forward primer: 5'-TGCTGAAAATGACTGAATATAAACTTGT G-3', reverse primer: GCTGTATCGTCAAGGCACTCTTG) and 1.25 U FastStart Taq Gold DNA polymerase. The size of the second COLD-PCR amplicon was 75 bp, and the cycling conditions were as follows: 95°C for 180 sec; 10 cycles at 95°C for 15 sec and 67.5°C for 50 sec; and 50 cycles at 80°C for 1 sec and 67.5°C (fluorescence reading on) for 60 sec in an ABI StepOne™ instrument. Experiments were repeated at least three times independently.

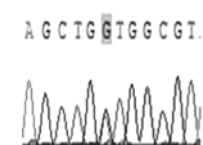
Statistical Analysis. All statistical analyses in the study were conducted using SPSS 13.0 software. The relationship between KRAS mutations in the plasma and matched tumor tissue, as well as KRAS mutation sequencing in the tumor tissue between regular PCR and COLD-PCR, were analyzed using the χ^2 test, with a P-value of <0.05 as a bilateral indicator of a statistically significant difference.

Results

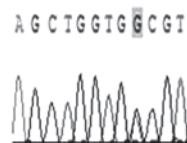
Comparison of KRAS mutation detection using regular PCR/sequencing and COLD-PCR/sequencing on tissue specimens. KRAS mutations were analyzed in the 62 specimens by direct sequencing. Twelve (19.4%) tissue specimens were considered to have the KRAS mutation (GGT>GAT) by regular PCR and COLD-PCR. The wild-type sample did not contain mutations, as detected by either method of amplification. A more pronounced mutation enhancement was obtained by COLD-PCR than by regular PCR for the same tissue specimens (Fig. 1).

KRAS mutations in plasma are correlated with mutations detected in the matched tumors. Thirteen (21.0%) KRAS mutations (GGT>GAT) were detected from the 62 plasma specimens whereas 12 (19.4%) KRAS mutations were found in the paired tumor tissues. Notably, four (6.5%) patients with

Regular PCR 165 bp KRAS seq

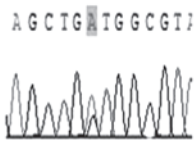


codon 12(GGT>GAT)

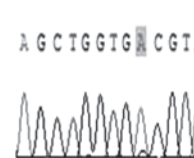


codon 13(GGC>GAC)

COLD-PCR



codon 12(GGT>GAT)



codon 13(GGC>GAC)

Figure 1. Mutations were detected by regular PCR, and COLD-PCR was also performed on the same specimens for comparison. COLD-PCR was found to improve the sensitivity of the sequencing.

Table I. Correlation of the KRAS mutation (GGT>GAT) in plasma and matched tumor tissues.

	Tissue		Total
	KRAS ⁻	KRAS ⁺	
Plasma			
KRAS ⁻	46	3	49
KRAS ⁺	4	9	13
Total	50	12	62

k=0.649; P<0.001.

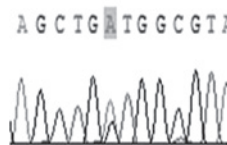
plasma DNA mutations had no detectable KRAS mutation in the corresponding tumor DNA specimens, and three (4.8%) patients with KRAS mutations in tumor DNA specimens had no detectable KRAS mutation in the corresponding plasma. The consistency of KRAS mutations between plasma and tumors was 75% (9/12), which indicated a high correlation between the mutations detected in plasma DNA and the corresponding tumor DNA (P<0.001; correlation index, k=0.649). The correlation between mutations detected in the plasma and tumors is summarized in Table I.

Discussion

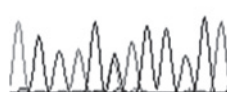
Several studies have already reported the presence of free circulating DNA in the plasma of cancer patients, exhibiting the same characteristics as primary tumor DNA such as oncogene expression, tumor-suppressor gene mutations, microsatellite and epigenetic alterations (11). Although the mechanism leading to the presence of free tumor DNA in the

A

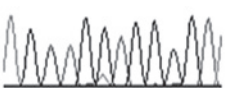
Mutations(GGT>GAT) in tumor



AGCTGTTGGCGT

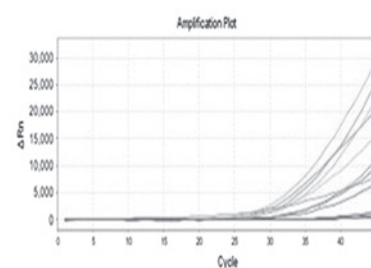


AGCTGGTGGCGT



B

A nested COLD-PCR/TaqMan-MGB in plasma



C

regular PCR/TaqMan-MGB probe in plasma

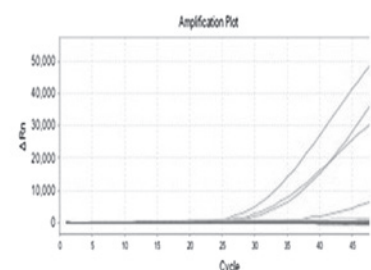


Figure 2. (A) Mutations were detected by COLD-PCR/sequencing in tumor DNA. (B) The same sample was amplified by the COLD-PCR/TaqMan-MGB probe for comparison. (C) A markedly pronounced difference in fluorescence was apparent between the conventional PCR and COLD-PCR results.

plasma of cancer patients is still not fully understood, it may be caused by the lysis of circulating cancer cells or by DNA leakage resulting from tumor necrosis or apoptosis (12). A number of studies have investigated the association of gene mutations between plasma or serum-free DNA and tumor tissues, and their clinical significance in cancer (13,14).

In our study, we found 13 of 62 (21.0%) patients with CRC had the KRAS mutation (GGT>GAT) in their plasma using the nested COLD-PCR/TaqMan-MGB probe, and 12 (19.4%) KRAS mutations were found in the paired tumor tissues by direct sequencing and the nested COLD-PCR/TaqMan-MGB probe, which was a positive correlation. A high consistency of the KRAS mutation between the plasma and paired tissue DNA was observed, which is consistent with studies by Yung *et al* and Maheswaran *et al* (15,16). Notably, four (6.5%) patients with plasma DNA mutations had no detectable KRAS mutation in the corresponding tumor DNA specimens, which may be attributed to the heterogeneity of the tumor cells. Only small samples of tumor tissue were used in this experiment, which may have been the portions without KRAS mutations in the tumor cells. In contrast, DNA in the plasma was released from different parts of the tumor, so in the plasma KRAS mutations could be detected. Three (4.8%) patients with DNA mutations in the tumor specimens had no detectable KRAS mutation in the corresponding plasma, which is possibly explained by the lower tumor cell content in some of the tumors contributing to the lack of detectable mutations in plasma. That the tumor areas carrying mutations shed less DNA than the other parts of the tumors into plasma may also be a reason for the lack of detectable mutations in the plasma.

Compared with regular PCR, the enhancement using COLD-PCR enabled clear detection of the mutation by direct sequencing (Fig. 1) and the TaqMan-MGB probe, which is consistent with studies by Mancini *et al* and Zuo *et al* (17,18). We also demonstrated that the KRAS mutation in the plasma DNA was not detectable using the regular PCR/TaqMan-MGB probe, since the level of the KRAS mutation in the plasma DNA was extremely low; only four samples that may have released a large quantity of mutant DNA into plasma appeared on the positive amplification curve (Fig. 2). We applied a nested COLD-PCR/TaqMan-MGB probe to detect KRAS mutations in the plasma. The first round of COLD-PCR increased the concentration of mutant alleles, and the second round of COLD-PCR further increased the concentration of mutant alleles detected by the TaqMan-MGB probe (Fig. 2). We found that the Tc played an important role in the enhancement of mutations during COLD-PCR. Using a Tc lower than 81°C further enhanced the relative proportion of mutant alleles for direct sequencing, however, this reduced PCR efficiency, made it more difficult to interpret the amplification curve when the Tc was lower than 80°C for the second nested COLD-PCR/TaqMan-MGB assay.

We know from previous studies that COLD-PCR/HRM is a convenient and sensitive method for identifying mutations (1,19), however, the specialist equipment required puts it beyond the reach of many hospitals. The COLD-PCR/TaqMan-MGB probe approach can be carried out using a real-time quantitative PCR instrument, which is relatively simple and cost-effective, so it may be widely used to detect point mutations in tissue and plasma, and plasma DNA analysis provides a noninvasive means of assessing KRAS mutations. We used fast-COLD-PCR during the experiment, since the method is rapid and the results are quickly obtainable (2 h for the nested COLD-PCR/TaqMan-MGB assay).

Although this method has numerous advantages, it also has limitations in that the results are false-negative when there are tumors with multiple mutations (codon 12 and 13 mutations). We detected KRAS mutations in the plasma during the experiment, but the experimental sample of 62 cases was limited, which may have resulted in bias. As such, further studies with a greater sample size and multi-point detection are required to further validate our results. In conclusion, KRAS mutations in plasma DNA correlated with the mutation status in the matched tumor tissues of patients with CRC. Our study provides evidence to suggest that plasma DNA may be used as a potential sample for KRAS mutation analysis in CRC using the COLD-PCR/TaqMan-MGB probe, particularly when tissue specimens are unable to be obtained. The COLD-PCR/TaqMan-MGB probe is a convenient, sensitive and cost-effective method for the detection of mutations and may have broad application for detecting mutations in a wide range of clinical settings.

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