Colorectal cancer (CRC) is one of the most common malignancies and one of the leading causes of cancer-related death in the developed countries. Distant metastasis is the main cause of death in CRC patients. Surgical resection remains the potentially curative option for patients with metastatic CRC (mCRC). However, the prognosis is poor as the curative resection is possible in less than 25% of patients with stage IV disease, and less than 5% of patients with unresectable mCRC are alive after 5 years. Major efforts are being made to improve the prognosis for patients with mCRC, and especially to develop new therapeutic strategies.

Monoclonal antibodies (mcAbs) (cetuximab and panitumumab) that target the epidermal growth factor receptor (EGFR) have recently been introduced for use as single agents or in combination with other chemotherapeutic drugs for the treatment of mCRC. However, the mcAbs only benefit a subset of cases that express the wild-type KRAS protein; tumors with mutated KRAS do not respond to this treatment modality. Thus, it is important that the KRAS mutation status be precisely determined to maximize the patient's benefit in a clinical setting.

Rapid and sensitive detection of KRAS mutation is needed to maximize the benefits for patients who are being treated with monoclonal antibodies to target the epidermal growth factor receptor in colorectal cancer. The aim of this study is to evaluate the efficacy of the peptide nucleic acid clamp real-time PCR (PCqPCR) as compared to that of direct sequencing (DS) between using fresh colorectal cancer tissue and the matched formalin-fixed and paraffin-embedded (FFPE) colorectal cancer tissue.

**Methods:** The efficacy of PCqPCR was evaluated and compared with that of DS using fresh tissue and matched FFPE tissue from 30 cases of colorectal cancer. PCqPCR detected 1% of mutants in 1 ng DNA. PCqPCR detected mutation in 1% of mutant cells, while DS barely detected, by manual reading, that in 20-50% of mutant cells. In the clinical samples, PCqPCR detected KRAS mutation in 60.0% while DS detected KRAS mutation in 53.3% of the colorectal cancers. The two methods showed a 100% concordance rate for detecting KRAS mutation between the fresh tissue and FFPE tissue.

**Conclusions:** The PCqPCR method is efficiently applicable for the detection of KRAS mutation in a clinical setting.
The peptide nucleic acid (PNA)-based PCR procedure has recently been developed for the enrichment of mutant alleles. Two important features make PNA a superior PCR clamp for specific alleles. PNA cannot serve as a primer for polymerization, nor can it be a substrate for the exonuclease activities of Taq polymerase. In addition, the melting temperature (Tm) of a perfectly matched PNA-DNA duplex is higher than that of the DNA-DNA of the same length, and a single mismatch destabilizes the PNA-DNA hybrids, causing a Tm shift of 10-18°C. Therefore, a PNA probe can specifically block chain elongation on a perfectly matched wild template without interfering with these reactions on the mutant templates with mismatched bases, and this enables the detection of mutants by real-time PCR.

Neutral buffered 10% formalin-fixed and paraffin-embedded tissue (FFPE) is mostly used in clinical practice. The formaldehyde modifies the nucleic acids, resulting in the addition of mono-methylol groups to the nucleic acid bases and also subsequently in the formation of methylene bridges between the nucleic acid bases and in nucleic acid-protein cross-links. FFPE is used widely for the detection of point mutation even if base damage by formalin fixation hampers the detection of point mutants. When applying a new method of mutant detection in the clinical setting, it is reasonable to test the reliability and efficacy of the new method as compared to the reliability and efficacy of the gold standard, which has been used so far.

We compared the efficacy of two platforms, peptide nucleic acid clamp real-time PCR (PCqPCR) and direct sequencing (DS), for the detection of KRAS point mutation using cell lines and we evaluated the KRAS mutant detection rate and the detection concordance rate between fresh frozen sectioned tissue and matched FFPE tissue using 30 cases of colorectal cancer.

To the best of our knowledge, this report is the first one that has compared fresh tissue and the matched FFPE tissue in mutational analysis of KRAS in colorectal cancer.

MATERIALS AND METHODS

Cell culture

To validate the sensitivity and specificity of the commercial PNA Clamp™ KRAS mutation detection kit (Panagene Ltd., Daejeon, Korea) as compared with DS for the mutational analysis of KRAS, three human cancer cell lines (HeLa, SW480, and LoVo) were used as controls for the PNA clamping standards for quantitative PCR. HeLa cells (Korea cell line bank No. 10002; KRAS wild type) were grown in DMEM medium (HyClone, Thermo, Logan, UT, USA). SW-480 cells (Korea cell line bank No. 10228; KRAS mutation c.35G>T: codon 12 GGT>GTT; p.Gly12Val) and LoVo cells (Korea cell line bank No. 10229; KRAS mutation c.38G>A: codon 13 GGC>GAC; p.Gly13Asp) were grown in RPMI 1640. The media were supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA) and this was used to cultivate the cells under standard conditions (humidified atmosphere, 5% CO2 in air, 37°C).

Patient samples

Thirty cases of surgically resected colorectal cancer were included in this study. Informed consent was obtained from the patients for the collection of the tumor specimens, and the study protocol was approved by the Soonchunhyang University, College of Medicine Ethics Committee. Tumor and normal tissue from the surgically resected specimens were frozen sectioned at 10 µm. The normal mucosa and inflamed areas were trimmed from the frozen block of tumor tissue as much as possible before frozen sectioning and extracting the DNA, and the remaining tissue after frozen sectioning was fixed in 10% neutral buffered formalin for the matched FFPE tissue block.

Preparation of genomic DNA

Genomic DNAs were extracted from the cell lines using the high pure PCR template preparation kit (Roche, Mannheim, Germany), according to the manufacturer’s instructions, and the DNAs were diluted to a concentration representing 50 ng/µL for the test. DNA from the SW-480 cells was diluted with the DNA from the HeLa cells to give mutation/wild-type ratios of 100%, 50%, 20%, 10%, 5%, and 1%. The DNAs extracted from the cell lines were stored at -20°C until use. The genomic DNAs from the frozen sections and the FFPE sections were extracted from 2 × 10 µm sections using the QIAamp DNA mini...
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kit and the QiAmp DNA FFPE tissue kit (Qiagen Inc., Valencia, CA, USA), respectively.

DNA sequencing

The PCR reactions were performed in a final volume of 50 μL that contained 1× PCR buffer, 200 μmol/L dNTPs, 200 nmol/L of each primer and 2.5 U of Taq polymerase (Solgent, Daejon, Korea). The forward primer was 5′-GTGGAGTATTGATAGTGTTAACA-3′. The reverse primer was 5′-TGTATCAAAGAATGGTCTCTGCA-3′. PCR was performed in a C1000™ Thermal Cycler system (Bio-Rad, Hercules, CA, USA) with the initial denaturation at 95°C for 5 minutes followed by 35 cycles of 1 minute at 95°C, 1 minute at 63°C and 1 minute at 72°C, and a final extension at 72°C for 15 minutes. The amplicons of 253 bp size were purified using the PCR purify kit according to the manufacturer’s instructions (COSMO Co. Ltd., Seoul, Korea). In brief, the amplicon was mixed by adding 5 volumes of PB buffer and the sample was loaded onto a column. After washing with NW buffer, the DNA was eluted with Tris buffer (10 mM Tris-Cl [pH 8.5]). DNA sequencing was performed with the PRISM 3100 DNA analyzer (Applied Biosystems, Foster City, CA, USA) using the ABI Prism BigDye Terminator version 3.1 (Applied Biosystems) with the forward primer.

PNA-based real-time PCR clamping

KRAS mutation was tested by using the PNA Clamp™ KRAS mutation detection kit (Panagene Ltd.) according to the manufacturer’s instructions. Briefly, PCR amplification was performed in a total volume of 20 μL that contained 50 ng of DNA, 13 μL of real-time SYBR Green PCR master mix and each of the primers and PNA probes for codons 12/13, respectively. The PCR control lacked a PNA probe. The PNA control contained the wild type template. The PCR cycling conditions were at 94°C for 5 minutes, followed by 40 cycles of four-steps (94°C for 30 seconds, 70°C for 20 seconds, 63°C for 30 seconds, and 72°C for 30 seconds), and a final extension of 72°C for 5 minutes. The PNA probe was designed to hybridize completely to the wild-type KRAS allele. PNA probe hybridization securely inhibits the amplification of the wild-type KRAS allele, while the PNA/mutant-type allele hybrid is unstable due to base pair mismatch and therefore it does not inhibit Taq polymerase from extension. The threshold cycle (Ct) was automatically calculated from the PCR amplification plots in which fluorescence was plotted against the number of cycles. Delta-Ct values were calculated as the Ct value of the PCR with the PNA control minus the Ct value of the PCR of the samples. The higher delta-Ct value means that the mutant was efficiently amplified. The cutoff value of 2.0 was used for determining the presence of mutant DNA.

Statistical analysis and software

Statistical analysis was performed using SPSS ver. 17.0 (SPSS Inc., Chicago, IL, USA). The percentage agreement and kappa statistics were calculated for the number of mutations in the PNA clamping method and the sequencing method between the fresh tissue and FFPE tissue to assess the agreement. Percentage agreements were obtained by dividing the number of paired observations in the agreement cells by the total number of pairs.

RESULTS

Sensitivity and specificity of the PNA Clamp™ KRAS mutation detection kit

The sensitivity and specificity were determined by serial dilution of the mutant DNA ranging from 100% to 1% into each background wild type DNA (1-50 ng). The delta-Ct of the 1% mutant in the background of DNA of 1 ng, 10 ng, and 50 ng were 3.4, 4.04, and 3.66, respectively. The delta-Ct of the 1% mutant in the 1 ng DNA was larger than the cutoff value 2.0. The kit was able to clearly identify a mixture containing 1% mutant alleles in the background of small DNA (1 ng) (Fig. 1).

Comparison with direct sequencing

The serial diluted mutant cells (SW480) in the background of wild type cell (HeLa) were tested in comparison with sequencing. The delta-Cts of 1%, 5%, 10%, 20%, 50%, and 100% mutant were 3.97, 6.05, 7.17, 8.24, 9.48, and 10.67, respectively. The delta-Ct of the 1% mutant in the background of DNA of 1 ng, 10 ng, and 50 ng were 3.4, 4.04, and 3.66, respectively. The delta-Ct of the 1% mutant in the 1 ng DNA was larger than the cutoff value 2.0. The kit could be used to detect a mixture containing 1% mutant alleles in the background of small DNA (1 ng) (Fig. 1).

Comparison with direct sequencing between fresh tissue and FFPE tissue

The serial diluted mutant cells (SW480) in the background of wild type cell (HeLa) were tested in comparison with sequencing. The delta-Cts of 1%, 5%, 10%, 20%, 50%, and 100% mutant were 3.97, 6.05, 7.17, 8.24, 9.48, and 10.67, respectively. The delta-Ct of the 1% mutant in the background of DNA of 1 ng, 10 ng, and 50 ng were 3.4, 4.04, and 3.66, respectively. The delta-Ct of the 1% mutant in the 1 ng DNA was larger than the cutoff value 2.0. The kit was able to clearly identify a mixture containing 1% mutant alleles in the background of small DNA (1 ng) (Fig. 1).
corresponding normal colonic mucosae by the kit or the sequencing either in the fresh tissue or in the FFPE tissue. The mutation of codons 12/13 was detected in 60.0% of the tumors by the kit, while the sequencing detected it in 53.3% of the tumors (Table 1). The sequencing failed to detect mutations in 2 cases. They were carcinomas with a heavy inflammatory reaction. To test the consistency rate of the number of mutations in the PC-qPCR and DS between the fresh tissue and the FFPE tissue, we calculated the overall percentage agreement rates and \( \kappa \)-values. The least agreement rate was 90.0% and the highest was 100%. The \( \kappa \)-values ranged from 0.967 to 1.000 for the consistency of the two methods, and all of the values were statistically significant (\( p < 0.05 \)). The two methods showed a 100% concordance rate between the fresh tissue and the FFPE tissue.

**DISCUSSION**

The presence of \( KRAS \) mutations in solid tumors can be detected by several different molecular methods. They are sequencing (direct sequencing, pyrosequencing) and allele-specific real-time PCR, and they each have their own merits and drawbacks. In a clinical setting, the test should be accurate, sensitive, have a short turnaround time and a low cost as well. Although DS has been used as the gold standard for the detection of mutation, it has low sensitivity. It can detect mutant allele when the sample contains above 20% of the mutant in the background of the wild type. The allele-specific real-time PCR requires more tissue for analysis as compared with other methods and the cost is high.

PNA is a synthetic DNA analog that does not occur in nature, in which the normal phosphodiester backbone is replaced with a 2-aminoethylglycine chain. Its nucleobases complement DNA or RNA in the normal A-T and G-C geometry and PNA is used widely as a probe for nucleic acids in molecular analysis of tumors. Single-base-pair mutation or single-nucleotide polymorphism analysis is possible using the PCR technique with a PNA probe, which complements to the mutant DNA. Various properties of PNA, e.g., the higher stability of a PNA/DNA duplex compared with the corresponding DNA/DNA duplex,
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the higher specificity of PNA binding to DNA and the inefficiency of PNA to act as a primer for DNA polymerases, form the basis for this technique. Basically, in the PNA directed PCR clamping technique, the PNA probe binding temperature set is higher than that for PCR primer binding. The PNA, which completely matches to the wild-type DNA, effectively blocks the formation of a PCR product. PNA is able to discriminate between fully complementary and single-mismatch targets (mutations) in a mixed target PCR because in the case of mismatches of PNA to mutant DNA, the PNA/DNA hybrid has a Tm much lower than that of the corresponding complete matches to wild DNA. Hence, the binding of the probe that completely matches to wild-type DNA will be favored to out-compete the binding of the mismatched PNA/mutant DNA. Consequently, the mutated sequences will be preferentially amplified. This PNA clamping is able to discriminate three different point mutations at a single position.

In PCqPCR, the PNA probe suppresses amplification of the wild-type KRAS sequence and therefore, this increases the sensitivity of detecting the mutant allele. Here we compared the sensitivity and specificity of the two platforms, DS and PCqPCR, for the detection of mutations in KRAS codons 12/13 using cell lines. To test the sensitivity of PCqPCR we used the DNA isolated from the known mutant cells line SW480, which is known to be homozygote, p.Gly12Val mutation and that from HeLa cells as the wild-type control.

We quantified the KRAS mutant alleles in the various mixtures (100%, 50%, 20%, 10%, 5%, 1%, and 0%) of the KRAS mutant cell line SW480 that was spiked in the wild-type HeLa cells, and we compared the results between PCqPCR and DS. The PCqPCR detected mutation in 1% of the mutant cells spiked in the wild type tumor cells, while DS barely detected, by manual reading, mutation in 20-50% tumor cellularity. The sensitivity of PCqPCR was much higher than that of DS. The DS was prone to give a false negative result depending on the amount of mutant cells in the sample. We tested the sensitivity of detecting KRAS mutant according to the input (template) DNA. We mixed the mutant DNA from the SW480 cells (1%, 10%, and 100%) in the wild type DNA with various total amounts of DNA as 1 ng, 10 ng, and 50 ng. The PCqPCR detected 1% mutant DNA in 1 ng of total DNA. The sensitivity of the limit of mutant KRAS detec-
tion in this study is in concordance with or is higher than that of other reports. Angulo et al. reported a detection sensitivity of 5% mutant DNA using the real-time PCR kit and Tsatis et al. demonstrated sensitivity of 5% in 10 ng by pyrosequencing. Dufort et al. detected 5% mutant DNA by nested PCR and pyrosequencing. Kobunai et al. reported a sensitivity of 0.4% in 2 ng DNA using cell lines and the PNA-clamp real-time PCR SYBR assay as a similar strategy to that in our study.

A sensitive method based on high-resolution melting analysis using a PNA clamp to characterize KRAS mutations was recently described. They reported the sensitivity was 0.1% in 100 ng DNA. Considering the input DNA, their sensitivity is not higher than that of this study, in which showed 1% detection sensitivity in 1 ng input DNA. Moreover, their procedure is not only cumbersome, but it also needs a sophisticated device that accurately controls the ramping temperature.

PCqPCR is very sensitive method to detect 1% mutants in a very small amount of DNA (1 ng), and it is suitable to test KRAS mutation with a small sample of biopsied tissue. In the clinical setting, small biopsy specimens that yield a low quantity of DNA are occasionally submitted for KRAS mutational analysis. The presence of KRAS mutations in plasma, serum, and urine has recently become a focus of interest. For these circumstances, the PCqPCR platform is the choice of method for KRAS mutational analysis of a low quantity of DNA. In this study, the KRAS gene of 30 samples of normal colonic epithelium was all the wild type either by DS or by PCqPCR. As the normal colonic mucosal epithelial cells do not harbor KRAS mutation, the test results of KRAS mutation indicate that the specificity of PCqPCR for testing KRAS mutation is 100%. The two platforms have their own advantages and disadvantages. DS examines the entire sequence and it can detect any type of mutation.
in the sequence amplified by PCR. The PCqPCR does not define a specific mutation. It was confirmed in this study that PCqPCR is more sensitive than DS for the detection of KRAS mutation. When we performed it, PCqPCR had a short turnaround time, about 90 minutes, and it required a lesser amount of hands-on time. Moreover, it is a closed system, which reduces the risk for contamination with amplicons carried over from the previous PCR reactions.

DS had a longer turnaround time and hands-on time than PCqPCR. DS identifies specific mutations and it can detect mutations outside of codons 12/13. However, it is much less sensitive to detect mutation than PCqPCR. Using an automated interpretation algorithm, it failed to detect mutation in 50% of mutant tumor cells and therefore all the DS data should be manually reviewed. The limit of detecting mutation by DS is subjective, and it may depend on the level of experience of the person interpreting the data. One benefit of this platform is its ability to evaluate a relatively long gene sequence for all possible mutations, although the clinical significance of KRAS mutations outside of codons 12/13 is unclear.23 The most common mutations outside of 12/13 are mis-sense mutations at codon 61, which occur in at least 1% of colon adenocarcinomas24 and its clinical relevance is unclear. For mutations outside of codons 12/13 and 61, the data indicating their relevance to patient management is limited, and so, clinical testing for this is not recommended at this time. Therefore it is not necessary to scan for KRAS mutation in the KRAS gene by DS. The PCqPCR platform can sensitively detect low levels of a mutant in a large amount of wild-type DNA. The clinical significance of low level mutants in relation to the prognosis and therapeutic benefit has yet to be fully studied. It is assumed that their identification will become clinically important. There is no currently significant data indicating that the specific mutation type affects the prognosis or the therapeutic choices. The American Society for Clinical Oncology suggests that pathologists use the terms "KRAS normal (nonmutated)" or "KRAS abnormal (mutated)" in their reports to facilitate the interpretation by the oncology community.25 Therefore, reporting the specific mutation in KRAS codons 12/13 is not clinically necessary. However, this may change as more data is accumulated.

The reported frequency of KRAS mutation has ranged from 29.6%26 to 65%27 depending on the methods. In this study, the KRAS mutation rate was 60% in the clinical samples by PCqPCR which was in close concordance with the rate reported by Span et al.21 (65%) with nested PCR followed by single-strand conformation polymorphism, and Tsatis et al.17 reported a KRAS mutation rate of 61.6% by pyrosequencing. However, the mutation rate in this study is much higher than 32.1% reported by Kobunai et al.19 who applied the same strategy that we used. They used 224 cases of frozen tissue for their study. The cause of such a different mutation rate may be possibly the small number of samples in this study.

FFPE is the clinical standard for processing samples in pathology departments. The huge archives of material stored in hospitals are invaluable resources for histopathology and molecular pathology, although formalin fixation results in the formation of chemical cross links between DNA/RNA and protein. Monomethylol groups are also added to nucleotide base pairs.28 These processes increase the susceptibility of the nucleotide sequences to shearing and fragmentation, and the DNA is often significantly degraded and present in short fragmented segments. Tests of gene mutations are occasionally hampered by these chemical changes of DNA by formalin fixation. When applying a new method of gene mutation, it is necessary to evaluate if the new method is applicable to FFPE by comparing the results of fresh tissue with the gold standard that has been used so far. To the best of our knowledge, there has been no report of gene mutation with comparing the result of fresh tissue and that of matched FFPE. The least agreement rate between the two platforms (PCqPCR and DS) was 90.0% and the highest was 100%. The \( \kappa \)-values ranged from 0.967 to 1.000 for the consistency of two methods, and all of the values were statistically significant (\( p<0.05 \)). As the two platforms showed a 100% concordance rate of KRAS mutant detection between fresh tissue and matched FFPE tissue, the PCqPCR is efficiently applicable to detect KRAS mutation in a clinical setting.

As shown in the mutant detection test with DNA from the mutant cell lines, the sensitivity of DS is lower than that of PCqPCR. DS failed to detect mutation due to the low percentage of the mutant allele of the tumor cells in the background of the wild type of inflammatory cells. This implies that the result of KRAS mutant detection by DS could be false negative depending on the mutant DNA fractions in the total DNA. To avoid false negativity by DS, the tissue that is to be subjected to mutational analysis must be examined by a pathologist to select the appropriate area from which the DNA should be extracted, and to determine the proportion of tumor cells. As shown, the minimum percentage of mutant DNA should be greater than 20-50% if DS is to be used.

In conclusion, we have shown that PCqPCR method is highly sensitive and accurate for the clinical analysis of KRAS mutation either in fresh tissue or matched FFPE with a 100% con-
cordance rate. This assay is directly applicable to FFPE clinical samples. This method is more sensitive than DS to detect small amounts of mutant DNA in abundant non-tumorous wild-type DNA. It has many advantages over DS such as its rapidity, sensitivity, accuracy and low cost. The sensitivity of this assay highlights the needs to clarify the association between the efficacy of the EGFR monoclonal drugs and low levels of mutant KRAS alleles, and it points to important new fields of clinical study.

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**REFERENCES**


