Using 2-Step PCR and Restriction Endonuclease Digestion to Detect *K-ras* Mutation in Paraffin-Embedded Tissues: Is It Reliable?

Wu-Chou Su,¹ Nan-Haw Chow,² Chia-Huy Huang,¹ and Hsiao-Sheng Liu^{3*}

¹Department of Internal Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan

²Department of Pathology, College of Medicine, National Cheng Kung University, Tainan, Taiwan

³Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, Tainan, Taiwan

In this study, we detected the existence of both wild type and mutant *K-ras* DNA on the cutting board and in the solutions for tissue processing in our pathology laboratory. The detection rate of *K-ras* mutant DNA inversely correlated with the frequency of the solution changed, implying that DNA contaminant including *K-ras* mutant may be accumulated while increasing specimens were processed. The exact routes of contamination during tissue processing remain to be determined. Nevertheless, this study unveils the possibility of DNA

contamination in the laboratory that can be amplified by PCR analysis, and precaution should be taken while we interpret our PCR data from paraffin-embedded specimens. The work also highlights the necessity of contamination control procedures, including specific laboratory construction, environmental control, and strict hygiene standards for laboratory equipment and personnel. Moreover, fresh specimens seem more reliable than archival materials for PCR diagnosis. *J. Clin. Lab. Anal.* 12:168–171, 1998. © 1998 Wiley-Liss, Inc.

Key words: PCR; *K-ras* mutation; PCR/RFLP

INTRODUCTION

Polymerase chain reaction (PCR) is a powerful technique whose popularity is increasing in molecular biology. The major advantages of PCR are rapid, high sensitivity and minimal sample requirement. Specifically, PCR techniques have been utilized to detect pathogens, mutations of oncogenes, and aberrant genes of many genetic diseases (1,2). However the exquisite capacity for amplification was accompanied by an extreme sensitivity to the presence of its own product as a feedback contaminate. In this work, contamination is defined as the unwanted presence of DNA (or RNA) amplicons that may sense a further in vitro amplification reaction (3).

Previous studies have reported the application of a 2-step PCR plus restriction enzyme digestion (PCR/RFLP) in the detection of *K-ras* gene mutation at amino acid codon 12 in biliary tract cancer (4–9). Our data show that approximately 89% of the patients with cholangiocarcinoma or ampullary cancer were *K-ras* mutation positive (unpublished data). The incidence of *K-ras* mutation in cholangiocarcinoma reported varied from 8.3% to 100% by the analysis of PCR, 2-step PCR plus RFLP, or 2-step PCR plus DNA sequencing (5,6,7,9,10). Such a large discrepancy in the *K-ras* mutation rate is either caused by the sensitivity difference of the analysis used or by PCR contamination. It is possible that some of our paraffin-embedded speci-

mens were contaminated by *K-ras* mutation DNA during specimen preparation and processing. To test this, we first determined the sensitivity of 2-step PCR/RFLP analysis for detecting the *K-ras* mutant then checked possible contamination sources during tissue processing.

MATERIALS AND METHODS

Sample Sources

SW480, a human colon adenocarcinoma cell line containing *K-ras* oncogene with a mutation at amino acid 12, was selected to be the positive control. A series of samples were taken in our pathology laboratory to investigate the possible sources of contamination that include the cutting board and tissue processing solutions (Table 1). All samples were processed using the “Hypercenter 2” tissue processing system and the “Varistain 24-3” automatic stainer (Shandon, Cheshire, Runcorn, England).

Grant sponsor: National Science Council, Taiwan; Grant numbers: NSC 85-2331-B006-29 and NSC 85-2331-B006-014.

*Correspondence to: H. S. Liu., Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, Tainan, Taiwan, R.O.C. E-mail: a713@mail.ncku.edu.tw

Received 7 November 1997; Accepted 10 November 1997

TABLE 1. Steps for Tissue Processing

Steps	Description
I: Cutting board	Cleaned and washed every day
II: Formalin (10%)	Change every day
III: Tissue processing	
Ethanol (85%, 95%)	Change every week
Xylene (1st & 2nd bottles)	Change every week
IV: Deparaffinization	
Xylene (1st & 2nd bottles)	Change every week
Ethanol (100%)	Change every week
V: Mounting	
Ethanol (70%, 95%, 100%)	Change every week
Xylene (100%)	Change every week

DNA Extraction

The samples (1.5 µl) were lysed with 200 µl of 4M guanidine-thiocyanate, followed by phenol/chloroform and chloroform/isoamyl alcohol extraction. The supernatant was then precipitated with 100% ethanol, and the pellet was dissolved with 50 µl of double distilled water before using.

PCR Amplification

The primers used for the 1st-step PCR were K1A and K1B (Table 2). The PCR reaction was conducted in a thermocycler GeneAmp 9600 (Perkin-Elmer Cetus, Emeryville, CA). The PCR mixture contains 100 ng DNA template, 1 µM each of primers, 200 µM dNTP, 1.5 mM MgCl₂, and 0.2 unit *Taq* enzyme. The total volume is 50 µl.

A nested PCR (2nd-step PCR) was performed using K1A and K1C as primers (Table 2) to amplify the 1st-step PCR DNA. The PCR reaction condition is 94°C for 2 min, 35 cycles of 94°C for 30 sec, 55°C for 2 min, and 72°C for 1 min followed by 72°C for 7 min. A PCR fragment of 157 base pairs (bp) would be amplified encompassing the amino acid codon 12 of *K-ras* gene. It was then digested with restriction endonuclease *Bst*NI (Biolabs, Beverly, MA) at 60°C for 2 h. The products of digestion were examined by electrophoresis on a 3% NuSieve gel (FMC, Rockland, ME) at 100 volts for 1 h.

DNA Sequencing

The PCR DNA was purified by QIAquick Spin PCR purification kit (Qiagen, Chatsworth, CA). A total of 5 µl of purified DNA was submitted for sequencing using a Sequencing PRO kit (Toyobo, Kitaku, Osaka, Japan).

TABLE 2. Oligonucleotide Primers Used for PCR

Primer	Sequence (5'-3')
K1A(+) ^a	ACTGAATATAAACTTGTGGTAGTTGGACCT
K1B(-)	TCAAAGAATGGTCCTGGACC
K1C(-)	GCATATTAACAAGATTAC

^a+, sense sequence; -, anti sequence.

RESULTS

The Sensitivity of 2-Step PCR/RFLP for Detecting K-ras Gene Mutation

To detect DNA contamination, we first optimized the PCR condition for *K-ras* mutation. The positive control DNA used was from SW480 cells, which contain a *K-ras* mutation at amino acid 12, and was diluted from 100 ng to 0.01 ng at a 10-fold dilution. The 1st-step PCR, using K1A and K1B as the primer pair, was conducted to amplify a 157 bp fragment encompassing the amino acid codon 12 of *K-ras* gene. Subsequently, the amplified fragment was digested with the restriction endonuclease *Bst*NI at 60°C for 2 h. *Bst*NI cuts the amplified wild type fragment twice and generates a fragment of 114 bp (data not shown), but cuts *K-ras* amino acid 12 mutant only once and generates a product of 143 bp (Fig. 1A,

Fig. 1. Detection of *K-ras* mutation in SW480 cells by 2-step PCR/RFLP. SW480 cell DNA was extracted by a rapid DNA extraction method. **Panel A:** the result of 1st-step PCR. Total DNA at the concentrations of 1000 ng (lane 3), 100 ng (lane 4), 10 ng (lane 5), 1 ng (lane 6), 0.1 ng (lane 7), and 0.01 ng (lane 8) was amplified by 1st-step PCR and digested with restriction endonuclease *Bst*NI. **Lane 2:** 100 bp maker, **lanes 1 and 9:** 10 ng of undigested control SW480 DNA, which shows the 157 bp uncutting fragments. The detection of a 143 bp DNA fragment represents the existence of the *K-ras* mutant. Wild type *K-ras* shows as a 114 bp fragment after digestion (data not shown). **Panel B:** the result of the 2nd-step PCR followed by *Bst*NI digestion (lanes 2-8). Wild type *K-ras* after *Bst*NI digestion shows as a 100 bp fragment, and the mutant shows as a 129 bp fragment. **Lane 1:** the 100 bp maker, **lanes 2-5:** the control normal human blood DNA at the DNA concentrations of 100, 10, 1, and 0.1 ng; **lanes 6-9** are mutant SW480 DNA at the concentrations of 10, 1, 0.1, and 0.01 ng, which are correspondent to lanes 5, 6, 7, and 8 in panel A; **lane 10:** negative control.

lanes 3–5). To further enhance the signal of the mutant fragment, 1.5 μ l of the *Bst*NI digested products was amplified by a 2nd-step PCR. The mutant *K-ras* fragment after 2nd-PCR amplification by primer K1A and K1C could no longer be cleaved by *Bst*NI and was shown as a 129 bp fragment, whereas wild type *K-ras* fragment was further cleaved by *Bst*NI and shown as a 100 bp fragment (Fig. 1B, lanes 2–5). Our data show that the minimal concentration of SW 480 DNA needed to detect *K-ras* mutation by 1st-step PCR was 1 ng DNA (Fig. 1A, lane 5). The minimal concentration required to detect *K-ras* gene mutation by 2nd-step PCR was 0.01 ng DNA (Fig. 1B, lane 8). The detection sensitivity of 2nd-step PCR is 100-fold higher than that of 1st-step PCR.

Possible Contamination Sources During Tissue Processing

The possible contamination sources investigated were the cutting board and solutions for tissue processing as well as for specimen preparation. To check the contamination on the cutting board, we washed the surface of the board with double-distilled water, and collected the water. The other samples were collected from the working solutions during tissue processing and specimen preparation (Table 1). Each sample was analyzed by 2-step PCR/RFLP analysis to detect *K-ras* mutation.

Figure 2 shows the fragment of the 2-step PCR after *Bst*NI digestion. An intensive 129 bp mutant fragment (over 60% of the total intensity) and a faint 100 bp wild type fragment were detected in samples from 95% ethanol (lane 8) and xylene solution (lane 9) of the tissue processor. In contrast, a weak 129 bp mutant band (at 50% or less of the total intensity) and intensive wild type 100 bp fragments were detected in samples from 85% ethanol (lane 4) for tissue processing, 100% ethanol (lane 10) for deparaffinization, and from 70% ethanol, 95% ethanol and xylene solutions (lanes 11, 12, and 13) for mounting (Table 1).

DNA sequencing was performed to verify the results of 2-step PCR/RFLP. Our data show that the sequences of the samples from the cutting board (Fig. 2, lane 2), 95% ethanol (Fig. 2, lane 8) and xylene (Fig. 2, lane 9) for the tissue processing contain a CAA mutation (wild type: CCA) (Fig. 3 and Table 3). In contrast, DNA sequences from the specimens showing less than 40% intensity of mutant fragment (Fig. 2, lanes 10–13) were wild type (Table 3), indicating that the weak 129bp mutant bands in these samples were incomplete *Bst*NI digested DNA. Moreover, very faint DNA was detected after PCR amplification from the samples of the cutting board and 10% formalin by 2-step PCR (Fig. 2, lanes 2 and 3). However, *K-ras* mutation was detected by DNA sequencing (Table 3), indicating the existence of *K-ras* mutant DNA contamination on the cutting board but at very small amounts. In conclusion, all the samples observed showed *K-ras* DNA fragment (including wild type and mutant), indicating the widespread contamination of genomic DNA in all the solutions checked. Moreover, the cutting board and some of the solutions used for tissue processing were further contaminated by *K-ras* mutant DNA in the pathological laboratory.

DISCUSSION

In this study, we demonstrated that paraffin-embedded samples prepared in the pathological laboratory through cutting, tissue processing, deparaffinization, and mounting procedures may be easily contaminated by various kinds of DNA (including mutant *K-ras*) because of the handling of diverse samples which were degraded during processing and DNA was released. Of particular interest was the inverse correlation between the detection rate of *K-ras* mutation and the frequency with which the working solution was changed. For example, the incidence of *K-ras* contamination in solution of ethanol or xylene (which was changed once a week) is much higher than in the solution of 10% formalin which was replaced every day. The results seem to imply that DNA con-

Fig. 2. Detection of *K-ras* mutation by 2-step PCR/RFLP. Sample DNA from each step of paraffin block preparation was amplified by 2-step PCR. The samples are from the following three parts: a) cutting board (lane 2), 10% formalin (lane 3), and tissue processor: 85% ethanol (lane 4), 95% ethanol (lane 8), xylene (lane 9); b) deparaffinization: 100% ethanol (lane 10); and c) mounting solution: 70% ethanol (lane 11), 95% ethanol (lane 12), xylene (lane 13). Lane 6: wild type control blood DNA. Lane 7: posi-

tive control of SW480 DNA. Lane 14: negative double distilled H₂O control. Lanes 1 and 5: 100 bp DNA makers. After *Bst*NI digestion, 129 bp uncut fragment represents mutant *K-ras* and 100 bp fragment represents wild type *K-ras*. The degree of *Bst*NI digestion of the PCR fragment was further classified into: 60% digestion: +++ (lanes 8 and 9); 50% digestion: ++ (lane 4); and 40% digestion: + (lanes 10–13).

Fig. 3. DNA sequencing of PCR fragments containing K-ras amino acid codon 12. PCR fragment for sequencing was initially purified by QIAquick Spin PCR purification kit and 5 μ l of the purified DNA was used for DNA sequencing, which was conducted by a Sequencing PRO kit. The sequence around amino acid codon 12 of K-ras was shown. The normal sequence of codon 12 is CCA. Blood: normal human DNA; SW480: DNA containing CAA mutation site; Lane 8 (Fig. 2): DNA from 80% ethanol of the tissue processor.

tamination, including K-ras mutant, was accumulated while the processing of degraded samples was increased.

The possible carry over contamination during pre-paraion of PCR reaction was ruled out by negative controls. The exact routes of contamination during tissue processing remain to be determined. Several possibilities may be considered for this issue. First, contamination may occur

through previous tissue debris that has been left on the cutting board, as demonstrated by the K-ras mutation from the board. Second, specimens may be contaminated during section using the unchanged knife or during paraffin embedding of the tissues through unchanged solutions. Third, some tissue fluid or tiny fragments from incompletely fixed samples may shed into solutions or containers during tissue processing.

Our observation is preliminary and awaits further investigation. At present, we can neither calculate the incidence of contamination nor the possible impact upon the interpretation of PCR analysis from paraffin-embedded material. Nevertheless, this result highlights the necessity of contamination-control procedures, including specific laboratory construction, environmental control, and the strict hygiene inspection (11) of laboratory equipment and personnel. Moreover, it appears that, by the current study, fresh samples are more reliable than archival materials for PCR analysis.

REFERENCES

1. Aoki Y, Lee JC, Pillai S, Isselbacher KJ, Rustgi AK: Radiolabeled polymerase chain reaction assay for detection of *ras* oncogene point mutations in tumors. *Clin Chem* 40:705-709, 1994.
2. Becker Y, Darai G: Diagnosis of Human Viruses by PCR Technology. Springer-Verlag, New York, 1992.
3. Kitchen PA, Szotoryi Z, Frombole C, Almond N: Avoidance of false positives. *Nature* 344:201, 1990.
4. Kondo H, Sugano K, Fukayama N, et al.: Detection of point mutations in the K-ras oncogene at codon 12 in pure pancreatic juice for diagnosis of pancreatic carcinoma. *Cancer* 73:1589-1594, 1994.
5. Levi S, Urbano-Ispizua A, Gill R, et al.: Multiple K-ras codon 12 mutation in cholangiocarcinoma demonstrated with a sensitive polymerase chain reaction technique. *Cancer Res* 51:3497-3502, 1991.
6. Tada M, Omata M, Ohto M: High incidence of *ras* gene mutation in intrahepatic cholangiocarcinoma. *Cancer* 69:1115-1118, 1992.
7. Tada M, Omata M, Kawai S, et al.: Detection of *ras* gene mutations in pancreatic juice and peripheral blood of patients with pancreatic adenocarcinoma. *Cancer Res* 53:2472-2474, 1993.
8. Tada M, Yokosuka O, Omata M, Ohto M, Isono K: Analysis of *ras* gene mutations in biliary and pancreatic tumors by polymerase chain reaction and direct sequencing. *Cancer* 66:930-935, 1990.
9. Watanabe M, Asaka M, Tanaka J, Kurosawa M, Kasai M, Miyazaki T: Point mutation of K-ras gene codon 12 in biliary tract tumors. *Gastroenterology* 107:1147-1153, 1994.
10. Imai M, Hoshi T, Ogawa K: K-ras codon 12 mutations in biliary tract tumors detected by polymerase chain reaction denaturing gradient gel electrophoresis. *Cancer* 73:2727-2733, 1994.
11. Mifflin TE: Control of contamination. *Molecular Bio-Products, Inc.*, California, 1995.

TABLE 3. Detection of K-ras Amino Acid Codon 12 Mutation by BstN I Digestion and DNA Sequencing of 2-Step PCR Products

Lane no. ^a	2	3	4	8	9	10	11	12	13	14
BstN I ^b digestion	-	-	++	+++	+++	+	+	+	+	-
CAA mutant ^c	y			y	y					
CCA wild type ^d		y	y			y	y	y	y	

^a Lane numbers are correspondent to those in Figure 2.

^b +++, over 60% BstN I undigested fragment; ++, 50% BstN I digestion; +, less than 40% BstN I digestion; -, no DNA fragment detected.

^c y, K-ras codon 12 CAA mutation detected.

^d y, K-ras codon 12 CCA wild type sequence detected.