Short Communication

Accuracy of DNA Amplification from Archival Hematological Slides for Use in Genetic Biomarker Studies[1]

Evelyn B. Boyle, Michael Steinbuch, Tanya Tekautz, Julie R. Gutman, Leslie L. Robison, and John P. Perentesis[2]

Children's Cancer Research Fund/Children's Cancer Group Molecular Epidemiology Laboratory and Divisions of Pediatric Hematology, Oncology, and Blood and Marrow Transplant, and Pediatric Epidemiology and Clinical Research, University of Minnesota Cancer Center, Minneapolis, Minnesota 55455

Abstract

Archival slides are a potentially useful source of DNA for mutation analyses in large population-based studies. However, it is unknown whether specimen age or histological stains alter the accuracy of Taq polymerase or induce secondary mutations in sample DNA. To address this question, we evaluated five methods for extraction of genomic DNA from archival bone marrow slides of 17 leukemia patients and analyzed exons 1 and 2 of the N- and K-*ras* genes for the presence of mutations. Of the five methods, optimal DNA purification was achieved by boiling and phenol:chloroform extraction. N- and K-*ras* exons 1 and 2 were independently amplified using 35 cycles of PCR, and 6–12 clones for each exon were isolated and individually sequenced for each patient. Mutations were confirmed by repeat extraction, cloning, and sequencing. Sixteen of 17 patient samples were successfully amplified (94%), including slides up to 29 years old. Twelve slides had been stained with Wright-Giemsa, 1 stained with toluidine blue, and 4 were unstained. A total of 16 single-base mutations were identified of 33,840 nucleotides sequenced. No insertions or deletions were identified. Six of 16 single-base mutations were previously described activating mutations in codon 13 of N-*ras* exon 1. The 10 other mutations were in other regions of the N- and K-*ras* genes and were not reproduced after repeat extraction, cloning, and sequencing. The frequency of these other alterations was 1 of 3384 bp. This value is comparable with the inherent error frequency for Taq polymerase. Our findings suggest that high fidelity DNA amplification can be achieved using archival hematological slides as old as 29 years and can be reliably used in genetic analyses.

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[2] To whom requests for reprints should be addressed, at Children's Cancer Group Molecular Epidemiology Laboratory, Room 554, University of Minnesota Cancer Center, 425 East River Road, Minneapolis, MN 55455.

Introduction

Archival material has been used for DNA mutation analyses involving various types of tissue. DNA has been successfully extracted from paraffin-embedded tissue, cytological smears (1, 2), cytogenetic slides (3), and Wright-Giemsa-stained hematological slides from bone marrow and peripheral blood (4–6). DNA from hematological slides, which were up to 10 years old, have been successfully amplified (4–6). Various methods have been used for DNA extraction from archived material (7–10). Several investigators have also successfully recovered RNA from formalin-fixed, paraffin-embedded tissue and hematological slides (11, 12). However, the poor yield and varying degrees of degradation of DNA and RNA from these sources of clinical material pose significant limitations for their consistent and reliable use in large scale studies. The extent of degradation has been shown to be related to the age, storage, fixation, and staining of the clinical material (12). In most circumstances, archival DNA template is unsuitable for analysis by traditional molecular techniques such as Southern blotting and cloning and other techniques that require large quantities of intact DNA.

The PCR (13–15) has proven to be an extremely powerful tool for the study of DNA and mutation analysis. PCR, which can produce a selective enrichment of specific DNA sequences by a factor of 10^6 (14, 15), has allowed the successful amplification and analysis of single human cells (16, 17). DNA from the various sources of archived material has been successfully amplified by PCR (reviewed in Ref. 18). Using PCR analysis of fixed stained tissue, it has been possible to type human papillomavirus (2) and to study translocations (19, 20) and loss of heterozygosity (21). One concern with using PCR for the amplification of archival material is the possible introduction of artifactual mutations in the template DNA during various fixation, staining, and storage techniques. In addition, it is unclear whether the fidelity of Taq polymerase is adversely affected by these same processes.

We have conducted a pilot study to assess whether DNA could be amplified from archived hematological slides from a group of 17 patients with leukemia. We have used the amplification and analysis of the N- and K-*ras* genes as a model for these investigations because *ras* is the most frequently mutated oncogene in leukemia, and the incidence and spectrum of N- and K-*ras* mutations are well defined. We first sought to define an optimal method for the efficient extraction of DNA template from archival slides. We then examined whether the procedures for staining and storing the slides increased the error frequency of Taq polymerase during PCR amplification. With this work, we have developed and characterized an efficient and high fidelity method to extract DNA from archival hematological slides.

Materials and Methods

Patient Samples. Stained (Wright-Giemsa or toluidine blue) and unstained archival bone marrow slides were obtained from

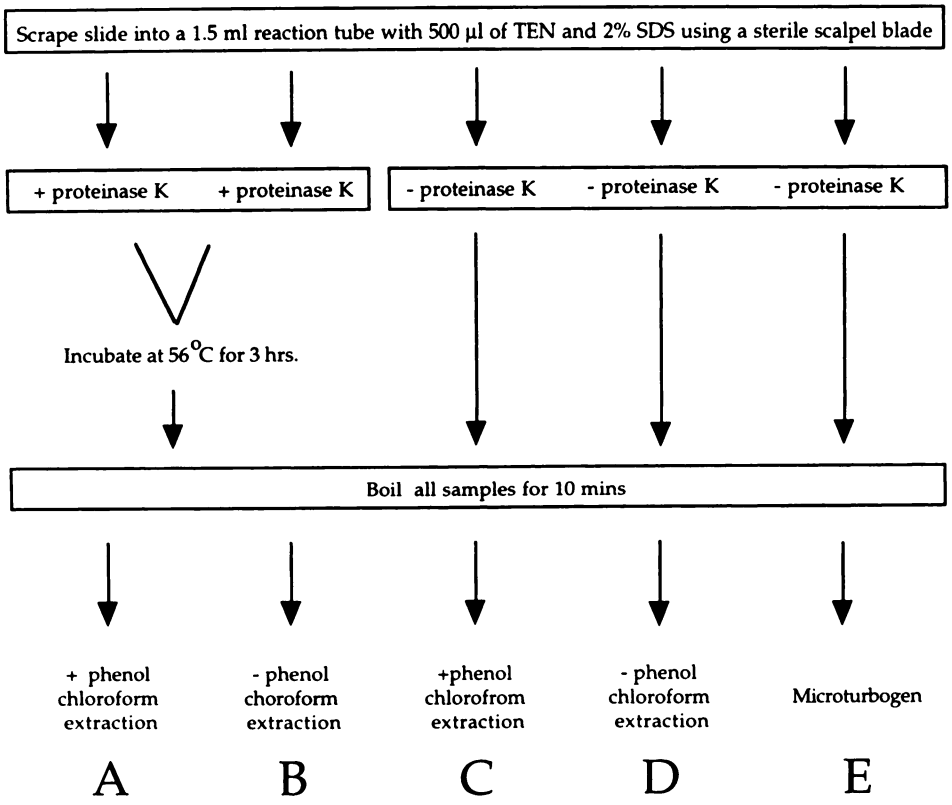

Fig. 1. DNA extraction methods from archival slides of bone marrow aspirate specimens.

the pediatric hematology laboratory at the University of Minnesota. A total of 17 bone marrow aspirates, one slide from each of 17 patients with leukemia diagnosed between 1967 and 1995, were studied. The 17 patients were selected for study because their archival files contained nonessential slides that were in excess of the number traditionally maintained in the laboratory, and the slides were otherwise intended for discard. The slides had been prepared between 1967 to 1995 and stored at room temperature. At the time of initial preparation, slides stained with Wright-Giemsa or toluidine blue had been fixed in methanol for 2 min before staining. Studies were approved by the Institutional Review Board: Human Subjects Committee of the University of Minnesota.

DNA Extraction from Stained and Unstained Hematological Slides. Five different methods for DNA extraction were compared in these studies and are outlined in Fig. 1. Four of these methods were designed to evaluate the relative contribution of proteinase K digestion (Fig. 1, Methods A and B) and/or an organic phenol/chloroform extraction (Fig. 1, Methods A and C) in sample preparation. In addition, one method involved the use of a commercial DNA purification resin (Fig. 1, Method E).

For each method, slides were initially scraped into 1.5-ml reaction tubes containing 500 µl of TEN [10 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0), and 0.1 M NaCl] using a sterile scalpel blade. To avoid cross-contamination between tubes, all procedures were performed in a sterile hood, and strict measures were observed to avoid PCR contamination. Once the material on each slide has been transferred to the buffer, the tubes were briefly centrifuged to force material adherent to the sides of the reaction tubes into the TEN solution. For cell lysis, SDS was added to a final concentration of 2%.

As summarized in Fig. 1, Methods A and B included the addition of proteinase K (50 µg/ml) with incubation at 56°C for 3 h. All of the methods then included boiling of the samples for 10 min. After boiling, the supernatants were transferred to clean 1.5-ml reaction tubes. Methods A and C then included a subsequent DNA organic extraction with phenol:chloroform (22). DNA was precipitated by adding 10 M ammonium acetate to a final concentration of 1 M and 2.5 volumes of 95% ethanol. Ethanol-precipitated samples were then stored at −20°C for a minimum of 2 h. DNA was pelleted by high speed microcentrifugation at 4°C for 20 min. The DNA pellet was washed once with 70% ethanol and then dried in a vacuum dryer for 3–4 min. DNA samples were resuspended in 50 µl of 10 mM Tris-Cl, 1 mM EDTA (pH 8.0). For Method E, DNA was extracted from one series of samples by using a commercially available genomic DNA extraction kit (Micro-TurboGen; Invitrogen). Template DNA prepared with each of these methods was then used to perform PCR.

The yield of DNA was measured using a Beckman DU spectrophotometer at 260 Å absorbance. Purified DNA templates were then used for PCR amplification of N- and K-*ras* gene exons 1 and 2, and cloning and sequencing of the amplified exons is described below. Two of the 17 patients also had a stored paired sample of genomic DNA frozen at −70°C that had been previously prepared directly from fresh leukemic bone marrow.

PCR, Cloning, and Sequencing of Samples. PCR was performed using oligonucleotide primer sets for K and N-*ras* exons 1 and 2, as described previously (23). PCR amplification was performed using 2.5 units of Taq polymerase (AmpliTaq or 2.5 units AmpliTaq Gold; Perkin-Elmer). Amplification reactions contained 150 nmol each of both 3′ and 5′ oligonucleotide

Table 1 PCR amplification and DNA sequencing analysis of N- and K-*ras* alleles from archival slides of bone marrow aspirate specimens

Specimen	Specimen age (yr)	Stain	Diagnosis[a]	Amplified *ras* allele(s)	bp sequenced	Initial mutation frequency estimate	Confirmed mutation frequency
H67-1086	29	Wright-Giemsa	AML, FAB-M7	K1	575	1/575	None
				N1	666	None	None
H75-1788	21	Toluidine Blue	"Congenital Leukemia"	K1	1727	None	None
H76-1287	20	Wright-Giemsa	AML	N1, N2	300	None	None
H77-1282	19	Wright-Giemsa	ALL, FAB-L1	K1	756	1/756	None
				N1	2517	1/1221	None
				N2	666	None	None
H78-405	18	Wright-Giemsa	DS-TMD	K1, N1	1197	None	None
H78-770	18	Wright-Giemsa	AML	K1, N2	1323	None	None
				N1	1332	1/1332	None
H83-2340	13	Wright-Giemsa	ALL, FAB-L1	K1	644	1/644	None
				N1	1332	None	None
H87-772	9	Wright-Giemsa	ALL, FAB-L1	K1, N1	2997	None	None
H89-701	7	Wright-Giemsa	DS-TMD	K1	666	1/666	None
				N2	1296	None	None
H89-1234	8	Wright-Giemsa	AML, FAB-M1	N1	888	1/888	None
H90-783	6	Wright-Giemsa	AML, FAB-M7	K1	1110	None	None
				N1	1887	2/1887	2/1887
H94-797	3	Wright-Giemsa	ALL, FAB-L1	K1	882	None	None
				N1	1415	4/1415	4/1415
				N2	1260	1/1260	None
HE95-301	1	Unstained	AML	N1	1665	1/1665	None
HE95-358	1	Unstained	ALL, FAB-L2	N1	2442	None	None
HE95-841	1	Unstained	DS-TMD	K1	1404	1/1404	None
				N1	999	None	None
LO3JT	1	Unstained	ALL	K1, N1	1894	None	None
H82-1862	15	Wright-Giemsa	AML-M7	None			
Total bases sequenced					33,840		
Total number of mutations						16	6

[a] AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; DS-TMD, Down syndrome transient myeloproliferative disorder; FAB, French-American-British classification.

primers and 0.1 mM deoxynucleotide triphosphates in a 50-μl total reaction volume. PCR amplification conditions included 35 cycles of 1 min at 95°C, 1 min at 56–62°C (depending on the T_m of the oligonucleotide primer pair), and 1 min at 72°C with a final extension step at 72°C for 10 min in a Perkin-Elmer thermocycler 480. When Taq polymerase was used, a hot start was performed for 5 min at 94°C. When using AmpliTaq Gold, enzyme activation was conducted for 12 min at 95°C. Samples were then cloned using the TA cloning kit (Invitrogen) and then transformed into bacteria. Plasmid clones were extracted and purified using the Perfect Prep kit (5 Prime 3 Prime, Inc.), and individual clones were sequenced using the AmpliCycle sequencing kit (Perkin-Elmer). Six to 12 plasmid clones were sequenced per sample, depending on the percentage blasts in the bone marrow. Mutations were confirmed by repeat PCR amplification from purified template DNA, followed by repeat subcloning and sequencing. For repeat specimens, the number of plasmids sequenced was equal to or greater than that sequenced after the first amplification.

To monitor for potential cross-sample DNA contamination of PCR samples, amplifications were also performed without DNA template. No amplification was detected from samples without template.

Results

Identification of an Efficient Method of DNA Extraction from Archival Hematology Slides. Five methods for DNA extraction were compared (Fig. 1). Successful PCR amplification was quantitated after separation on a 2% agarose gel stained with ethidium bromide (data not shown). The two methods (*i.e.*, Methods B and D) that did not include organic extraction after boiling did not consistently result in successful DNA amplification. Methods A and C, which included organic extraction, resulted in successful DNA amplification, as did the samples extracted with Microturbogen (Method E). Incubation with proteinase K (Method A) did not enhance the efficiency of DNA template purification in samples treated with organic extraction. Therefore, for our subsequent analyses, we chose to extract DNA by scraping the contents of the sample slides into 500 μl of TEN and 2% SDS, boiling for 10 min, followed by a phenol:chloroform extraction (Method C). Template DNA was recovered from archival slides using this method in amounts up to 2.4 μg/slide measured by spectroscopic absorption at 260 Å.

Fidelity of PCR Amplification from Archival Hematological Specimens. We next sought to estimate the fidelity of the boiling/organic extraction method by PCR amplification of N- or K-*ras* exons 1 or 2 from 17 archival slides. Table 1 details the diagnosis, age of slides, slides staining method, the number of bases sequenced, and the initial estimated and confirmed mutation frequencies per sample. Twelve slides were stained with Wright-Giemsa, one with toluidine, and four slides were unstained. In 16 (94%) patients, we successfully amplified, cloned, and sequenced at least one *ras* exon. One sample failed PCR amplification (H82-1862). Apart from this sample, the success of PCR amplification was not associated with the amount of DNA recovered, the age of the slide, or slide staining procedures (data not shown).

We estimated the mutation frequency by analyzing the DNA sequence of a total of 33,840 bases (Table 1). The total

Table 2 Confirmed mutations found after reamplification and sequencing of DNA template from archival slides

Specimen	Specimen age (yr)	Stain	Diagnosis	Amplified *ras* allele(s)	bp sequenced	Confirmed mutation
H90-783	6	Wright-Giemsa	AML, FAB-M7	N1	1887	codon 13 GGT to GAT gly to asp
H94-797	3	Wright-Giemsa	ALL, FAB-L1	N1	1415	codon 13 GGT to GAT gly to asp

" AML, acute myeloid leukemia; FAB, French-American-British classification; ALL, acute lymphoblastic leukemia.

number of N- and/or K-*ras* gene exon 1 or 2 bases sequenced for each patient was a function of the number of plasmid clones sequenced per sample and the number of readable bases per sequence. Our initial screen identified base substitutions in 16 of the 33,840 bases sequenced as an initial mutation frequency estimate (Table 1). No insertions or deletions were identified. Six of the 16 point mutations were a previously described activating mutation in codon 13 of N-*ras* exon 1. In two patients (H90-783 and H96-797), the same mutation was found in codon 13 (GGT/gly to GAT/asp) of N-*ras* exon 1 in six plasmids sequenced. In both of these samples, the mutation was found after at least two separate cycles of repeated PCR amplification and likely represent a molecular change in the leukemic blasts (Table 2). The 10 other mutations were in other regions of the N- and K-*ras* genes and were not reproduced after repeat extraction, cloning, and sequencing. The frequency of these other alterations, which most likely represent PCR artifactual mutation, was 1 of 3384 bp (Table 1).

Analysis of Paired Samples of Archival Slides and Frozen Genomic DNA. Paired samples of frozen genomic DNA that had been prepared by Ficoll separation, lysis, and phenol: chloroform extraction of fresh bone marrow mononuclear cells were available for two patients (H89-1234 and H94-797). These frozen genomic DNA samples were from the same bone marrow aspiration that was used to prepare the archival slides that were used in our study. Mutation analyses of archival slides and paired samples of frozen genomic DNA extracted from fresh bone marrow were in accord with one another in both of these patients (data not shown). In patient H94-797, analysis of both DNA extracted from bone marrow and the corresponding archived stained slide revealed an identical mutation in N-*ras* exon 1 at codon 13 (GGT/gly to GAT/asp). In patient H89-1234, no *ras* mutations were detected by analysis of either DNA freshly prepared and frozen at the time of bone marrow aspiration or DNA extracted from archival slides. These observations further support the notion that high fidelity DNA analysis can be conducted using archival stained slides.

Discussion

Large scale molecular epidemiology studies hold considerable promise in delineating the etiological mechanisms of specific malignancies, including leukemia. The use of archival hematological specimens in these studies offers potential advantages for cost-effective specimen acquisition and efficient high fidelity DNA mutation analysis. In our investigation of the spectrum of *ras* mutations in archival hematological slides, a total of 16 bp substitutions were identified of 33,840 nucleotides sequenced. Six of these mutations were a previously described activating mutation in codon 13 of N-*ras* exon 1. The 10 other bp substitutions were in other regions of the N- and K-*ras* genes and were not reproduced following repeat extraction, cloning, and sequencing. The frequency of these other alterations was 1 of 3384 bp.

The fidelity of Taq polymerase has been reported to be between 1×10^{-4} and 1×10^{-5} errors per base per PCR cycle (14, 15, 24–28). Errors were found with a frequency of 1 of 3384 bases in our cohort. The error rate in our cohort can be estimated by dividing the error frequency by the number of PCR cycles. The estimated error rate, 0.8×10^{-5} errors/base/PCR cycle, is within the range of that reported for Taq polymerase. Failure to confirm these mutations by reamplification and sequencing was an effective measure to assure fidelity of the results of this study. In total, these observations indicate that the staining and storage of slides is not significantly mutagenic and, with PCR amplification, does not increase the frequency of base misincorporation by Taq polymerase.

In further support of this conclusion, in a limited investigation, we observed that mutation analyses in the paired samples of archival slides and frozen genomic DNA were concordant. Paired samples were available in two patients, and one patient (H94-797) exhibited a commonly described activating *ras* mutation at codon 13 that was identified in both the archival slide and stored frozen DNA sample. A second patient had wild-type DNA sequence in both slide and frozen DNA. In addition, we did not observe mutations involving insertions or deletions in any patient, consistent with the previous reports of the extremely low frequency of such mutations in N- or K-*ras* (29). This preliminary observation awaits confirmation in larger prospective studies that compare mutation frequencies in paired samples of stored slides and frozen genomic DNA.

Prior to the use of PCR analysis, the study of DNA was limited by the fact that molecular biological techniques such as Southern blotting required large amounts of high grade genomic DNA. With the introduction of PCR, archival specimens are becoming widely used sources of DNA. Archived slides of bone marrow and blood samples are a powerful source of DNA for the molecular study of hematological diseases, as well as for constitutional genetic abnormalities. Our findings suggest that high fidelity DNA amplification can be achieved using archival hematological slides as old as 29 years and can reliably used in genetic analyses.

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