The Quality of DNA Recovered from the Archival Tissues of Atomic Bomb Survivors is Good Enough for the Single Nucleotide Polymorphism Analysis in Spite of the Decade-long Preservation in Formalin

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It is well known that the yield of DNA recovered form tissues preserved in formalin is inversely proportional to the stored duration. How is the quality? We tested the quality of DNA from archival tissues of atomic-bomb survivors stored in formalin for decades with the parameters of gene amplification efficiency by a polymerase chain reaction. All of the DNA extracted from the tissues preserved in formalin for 30 years amplified the 54- and 61-base pairs of the DNA fragments successfully. The direct sequencing of the PCR products confirmed the accurate amplification of the target sequence. A further trial to amplify the longer sequence of 111 base pairs succeeded in 20% of the samples tested. From these results, we propose a new utility of archival samples for the analysis of single nucleotide sequence polymorphism of genes, no matter how long the samples have been preserved in formalin.

INTRODUCTION

Archival tissues are bountiful resource for various studies. The polymerase chain reaction (PCR) benefited us to use tissues embedded in paraffin for the molecular analysis of disease1. Extracted DNA could be provided for all types of assays that required PCR amplification, restriction fragment length polymorphism, single-strand conformation polymorphism, and direct sequencing2,3. Concerning the paraffin-embedded tissues, the age of the samples was not a very great problem, as was the source of procurement.

The Research Institute for Radiation Biology and Medicine (RIRBM), Hiroshima University, has saved archival specimens of atomic-bomb survivors (ABS) for 5 decades. The specimens comprise the tis-
sues preserved in formalin, tissue blocks embedded in paraffin and blood smears. Among these, the number of tissues preserved in formalin (8044 cases in total by the end of 1997) is ten-times that of paraffin blocks. This is because an individual pathologist has kept paraffin blocks on his own in order to prepare for any kind of analysis. On the other hand, keeping the tissues in formalin has not been welcomed. First of all, it consumes space. Secondly, most scientists agree that it would be hopeless to extract high-quality DNA from tissues preserved in formalin for decades because of the fixative’s irreversible denaturing effects on DNA. After all, tissues in formalin, such as that left over from macro- and micro-pathological analyses, have been kindly transferred from pathologists to RIRBM, where they have been stored with no idea concerning their use. If the DNA could be recovered from the tissues in formalin, over 8000 samples of ABS would become a resource for data mining especially concerning the single nucleotide polymorphism (SNP) on the genome. Elucidating the relationship between genotype and phenotype, one SNP on a target gene and the susceptibility to radiation, for example, is one of the most challenging tasks of the 21st century. Much information about the exposed dose and the disease history after the atomic-bomb explosion for individual ABS is a strong right hand for SNP research.

The purpose of this study was to look for a utility of ABS’ archival tissues preserved in formalin for decades. After showing the nature of degrading DNA during preservation in formalin fixative using mouse tissues, the extraction of DNA from the ABS’s specimens was performed. We could satisfactory recover DNA for PCR analysis from livers preserved for over 3 decades.

MATERIALS AND METHODS

Source of mouse livers

Liver specimens from hybrid mice (C57BL/6N x C3H/He) were immersed in 4% formalin (pH 5.5) or 4% phosphate-buffered formalin (pH 7.4) for 1 to 21 days at room temperature or 4°C.

Source of archival specimens

Necropsy specimens of ABS fixed with the 4% formalin (pH 5.5) have been preserved in a storage room with temperatures between 5 and 18°C. The fixative was renewed every 3 years. Fifty liver specimens stored in formalin for 32 years, 15 for 20 years and 49 for 5 years were tested. Three fresh livers and 5 in vitro leukemia cell lines were used for unfixed control. The blood smear-specimens of ABS were aged control free from exposure to formalin. These samples were fixed with 100% methyl alcohol, stained with Giemza, glass-covered and stored in the same storage room for up to 55 years. Two samples of liver embedded in paraffin for 55 years were tested as being representative of short term-fixation in formalin.

DNA extraction by Shumidt-Thanhauser-Schneider’s (STS) method

The STS method was used to extract DNA quantitatively. Briefly, finely minced livers were made up to 20% (w/v) of a saline suspension. After removing acid-soluble phosphates, phospholipids and RNA by trichloro acetic acid, ethyl alcohol/ether (1:1, v/v) and perchloric acid, respectively, the fraction of DNA was extracted by perchloric acid at 100°C. The total amount of the extracted DNA was measured an optical density densidometer.

DNA extraction by the phenol/chloroform method

The phenol/chloroform method was used to extract DNA qualitatively. Liver tissues stored in formalin were embedded in paraffin. Thin sliced liver of 90 (15 x 15 x 0.4) mm³ per case was provided for DNA extraction. The paraffin, followed by the proteins, was removed before DNA extraction. Extraction in a buffer containing 500 µg/ml of proteinase K took up to 7 days at 37°C. The recovered DNA was dissolved in 20 µl of TE buffer (10mM Tris-HCl (pH7.4), 1mM EDTA).

Bone marrow cells (BM) and peripheral blood cells (PB) were scratched from the slide glass over a
20 × 40 mm²-area with a thickness of approximately 0.01 mm. Then 0.4 \((10 × 10 × 0.004)\) mm³ of paraffin-embedded liver tissues (Paraffin) was scratched from the slide glass. An extraction buffer containing the 100 µg/ml of proteinase K was treated on the samples over night at 37°C. The recovered DNA was dissolved in 20µl of TE buffer.

**DNA isolation by a rapid method**

A rapid DNA-isolation-agent (Wako Ltd., Japan) was used to isolate DNA from ABSi livers stored in formalin for 20 years. After 90 mm³ of liver preserved for 20 years was embedded in paraffin, it was thin-sliced for DNA isolation. The isolated DNA was dissolved in 20µl of TE buffer.

**Table 1.** List of primers used for qualitative assessment of DNA.

| Primer | (Indivisual name) | Priming gene | Sequence | Amplicon size (bp) |
|--------|------------------|--------------|----------|--------------------|
| 1      | (human-RAS-F)    | H-RAS        | AGACGTGCCTGTGGACATC |                   |
| 2      | (human-RAS-R)    | H-RAS        | CGCATGTAACGTGGTCCCGCAT | 110               |
| 3      | (mouse-ras-F)    | H-ras        | CAGTCCTACGGAAACAGCTC |                   |
| 4      | (mouse-ras-R)    | H-ras        | GGGAATACACAGAGGCCTTC | 147               |
| 5      | (F2917)          | REV1         | TCTACATACTCCTGTCAGTG |                   |
| 6      | (R2970)          | REV1         | GGGACCTCTATATCAGGTT | 54                 |
| 7      | (F3409)          | REV1         | CCATCGGTTCACAAAAAGG |                   |
| 8      | (R3469)          | REV1         | CAGGACTGTAAGCAGCTTG | 61                 |
| 9      | (F3339)          | REV1         | TGCCAAAGAATCCTTTACTTCATC |                   |
| 8      | (R3469)          | REV1         | CAGGACTGTAAGCAGCTTG | 111               |
| 10     | (PIGA-F)         | PIGA         | GGGAGAGAGTCAGATAATC |                   |
| 11     | (PIGA-R)         | PIGA         | GAGCTGACATCAGGAAATCC | 126               |

The condition for amplifying of the H-ras and H-RAS genes consisted of 1 min of denaturing at 94°C, 30 sec of annealing at 60°C and 1 min of extension at 74°C. The repeated number of each PCR was 30 cycles. The PCR products were separated on 10% acrylamide gel or 3% agarose gel.

The PCR products of the 54- or 126-base pairs (bp) of the REV1 or PIGA gene, respectively, were directly sequenced from the primers of number 6 and 10 (Table 1) using the ABI373 DNA sequencing system (Applied Biosystems).

**Statistical assessment**

The yield of DNA extracted by the STS method was examined statistically for significance using the t-test.

**RESULTS**

**Nature of degenerating DNA during preservation in formalin by mouse liver**

The effect of formaldehyde was shown on the yield of DNA extracted by the STS method (Fig. 1-A, B). The preservation temperature, duration in a fixative and pH of the fixative were the parameters. The yield of DNA decreased in a fixed duration-dependent manner. The samples fixed in 4% phosphate buffered formalin (pH7.4) at 4°C did not show
Fig. 1. Effects of duration stored in a fixative on the yield of DNA recovery by the STS method. a; Tissues were stored in the 4% formalin (pH5.5) at room temperature (●) or at 4°C (▲). b; Tissues were stored in the 4% phosphate buffered formalin (pH7.4) at room temperature (●) or at 4°C (▲). The decreasing rate for the yield of DNA was estimated from a linear fitting model.

Fig. 2. Effects of duration of storage in a fixative on the quality of DNA. The 147-bp-sequence of the H-ras gene was amplified for 1μg of DNA extracted from a mouse liver tissues fixed in 4% formalin. The fixation was performed at room temperature (A) or 4°C (B). Lanes 1 and 5, lanes 2 and 6, lanes 3 and 7, and lanes 4 and 8 were DNA from tissues fixed for 1, 7, 14 and 21 days in fixatives, respectively (10% acrylamide gel).
any decrease in the yield of DNA for up to the 21 days.

The PCR for amplifying the H-ras gene was applied to DNA extracted from murine livers fixed with buffered or unbuffered formalin, and preserved at room temperature or 4°C (Fig. 2-A, B). A specific band was seen at the 147-bp region in all of the PCR products.

**Yield of DNA extracted from the liver of ABS.**

Table 2. showed the yield of DNA recovered from by the STS method. The mean amount of DNA extracted from the tissues fixed in 4% formalin (pH 5.5) for 32 or 5 years was half as much as that from the unfixed tissues (p < 0.05). The yield of DNA from the tissues stored for 5 years was as much as that for 32 years. The yield of DNA from livers was significantly shorter than that from cultured leukemia cells (p < 0.01). The yield of DNA from the human livers was not different from that from mice.

**Quality of DNA extracted from the liver of ABS.**

The DNA extracted from the liver by the phenol/chloroform method was electrophoresed (Fig. 3). When 1µg of the extract was applied on the gel, the smear was visible in one out of 3 samples for each 32- or 5-year-fixed group (lane 8 of Figs 3-A and B). However, the smear disappeared in the case of 32-year-fixed case a RNase-treatment (lane 7 of the Fig. 3-A).

![Fig. 3.](https://academic.oup.com/jrr/article-abstract/43/1/65/910226)

Expecting a high efficiency of gene amplification, 2µl of extract was applied for the PCR of shorter amplon, the 54-bp-sequence of the REV1 gene (12). Thirty cycles of PCR amplified the 54-bp-fragment in 3 out of 5 cases for each group (lane 2, 3 and 7 of Fig. 4-A, and lane 2, 4 and 5 of Fig. 4-B). When 60 cycles were tried for PCR, the incidence of successful amplifications increased in all groups (Figs 4-C, D) (Table 3). Direct sequencing of the amplified fragments had the primary structure of the 14-bp-sequence between the two primers of 20-bp.
The 14-bp-sequence was identical to the genomic sequence of the REV1 gene.

A subsequent trial for 111-bp-amplification did not succeed by the first 30 cycles of PCR (upper panels of Figs 5-A, B). The band became visible after another 30 cycles of PCR in 1 out of 5 samples for each group (lane 7 of Fig. 5-A and lane 2 of Fig. 5-B). The final incidence of successful amplification was 20% (Table 3).

The amplifications of 54- and 111-bp-sequences were performed on DNA isolated from the 20-year-preserved ABS’ livers by a rapid method. The amplification succeeded 100% for the 54-bp fragment when 60 cycles were used for PCR (Table 3). However, it did not succeed to amplify the 111-bp-sequence even after 60 cycles of PCR (Figs. 6-A, B).
Quality of the DNA extracted from archival specimens of the BM, PB and Paraffin.

The results of PCR on DNA extracted from BM, PB and paraffin are shown in Fig. 7. In the cases of BM and PB, the cells had not been exposed to formalin at all. In the cases of paraffin, the tissues had probably been fixed in formalin for a couple of days before being embedded in paraffin. The DNA quality extracted by the phenol/chloroform method was good enough to amplify the 126-bp of the \textit{PIGA} gene (Fig.7-A). In the cases of the oldest samples of BM and paraffin testable in RIRBM, the first 30 cycles of PCR did not clearly show an amplified band (Fig.7-A). But the band became visible after another 30 cycles of PCR in all three samples (Fig.7-B). A sequence analysis indicated that the PCR products had the same sequence between the two primers, number 10 and 11 (Table 1).

A simple calculation helped us to estimate the yield of DNA, which was sufficient for gene amplification (Table 4). The DNA extracted from BM, PB or paraffin could amplify a sequence with a length of 126-bp, when about 660 or 30 ng of DNA was applied. However, the DNA extracted from liver tissues stored in formalin for 32 or 5 years required about 100µg to amplify sequences with lengths of 54-and 61-bp. The efficiency was about 1295–2850 times lower in the formalin-preserved groups than in
Fig. 6. Same amplification of a longer sequence as in Fig. 5 for DNA isolated by the rapid method. The 111-bp-sequence of the REV1 gene was amplified for 2 µl of DNA isolated from liver stored in 4% formalin (pH5.5) for 20 years. The PCR amplification was repeated for 30 (A) or 60 (B) cycles. Lane 1, marker; Lanes 2–6, DNA from the ABS' livers; Lane 7, DNA from a human leukemia cell line in panel A or marker in panel B; 3% agarose gel.

Fig. 7. The 126-bp-sequence of the PIGA gene amplified for 2 µl of DNA extracted from the bone marrow cells (BM), peripheral blood cells (PB) and liver tissues embedded in paraffin (Paraffin). PCR was repeated for 30 (A) or 60 (B) cycles. AFIP samples had been prepared at the Armed Force Institute of Pathology, USA, in 1945. 3% agarose gel.
unfixed groups.

**DISCUSSION**

Cross-linking between DNA and DNA or DNA and proteins can be induced by various chemicals and physical agents, many of which, including formaldehyde, are known or suspected to be carcinogens.\(^\text{13,14}\) DNA-protein cross-linking (DPC) involving all major histones is the dominant form of DNA damage in cells exposed to formaldehyde.\(^\text{15}\) Most of the DPC disappears through spontaneous hydrolysis or by being actively repaired in a live cell.\(^\text{16}\) However, DPC also occurs in cells after their death. The rate to form DPC is proportional to the concentration of formaldehyde.\(^\text{17}\) When the formaldehyde solution is acidic, hydrogen bonds between the pairing nucleotides dissociate to cause an irreversible denaturing of double-stranded DNA.\(^\text{18}\) Storing tissues at higher temperature accelerates the dissociation of hydrogen bonds.\(^\text{17}\) Therefore, as long as the tissues have been in formalin, the formation of DPC and its hydrolysis repeat to denature DNA.

Even though the length of fixation was within several hours, the recovery of DNA from tissues was reduced in amount.\(^\text{19}\) The speed to dissolve the tissues was delayed in the case that length of storage in formalin was over 5 days.\(^\text{20}\) In our experiment, decade-long preservation of tissues in formalin caused a difficulty to dissolve. The formation of the DPC complex was imaginable in such insoluble fractions. However, the incompleteness of tissue solubilization did not affect the yield of DNA; no difference was found in the amounts of recovered DNA between the groups stored for 5 years and that stored for 32 years (Table 2). Nearly half the number of phosphates was thought to be missing from the phosphate-deoxyribose backbone of the DNA strands. Some amount of phosphates must be lost along with deoxyribose as very short fragments of DNA during the quantity-measuring procedure. However, this does not explain the reason for the similarity in the amount of recovered DNA between groups stored for 32- and 5-years. There are two components in DNA denaturation; the DPC-formation and fragmentation. An experiment to measure the yield of DNA recovered from synthetic DNA, which does not contain any nuclear protein, is required to understand the effect of formalin on DNA fragmentation.

Decade-long preservation in formalin required about a 1295~2850 times larger amount of DNA for gene amplification compared to the amount of DNA recovered from the unfixed groups (Table 4). The acceleration of DNA fragmentation by formaldehyde seems not to be a big problem for the amplification of DNA.
short fragments of less than 100-bp. They could be overcome by increasing the cycle number of PCR, the amount of DNA extract for PCR application or the amount of tissues for DNA extraction at the beginning.

When we compared the way of recovering DNA, the rapid method was found to be less efficient than the phenol/chloroform method (Table 3). Because DNA isolated by the rapid method had to be concentrated by more than 1000 times than the concentration recommended by the industrial protocol, this method was not convenient for DNA isolation from the archival samples. The mechanism used to isolate DNA was to remove proteins from it with some kinds of detergents in the rapid method.21

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