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Technical Advance

Feasibility of Using Decades-Old Archival Tissues in Molecular Oncology/Epidemiology

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Archival tissues are a bountiful resource for various studies. Polymerase chain reaction permits the use of such tissues for molecular biological analyses of disease causation. However, a comprehensive study using a large number of decades-old samples (20 or more years) for molecular oncology/epidemiology has never been shown to be feasible. We have relied upon the unique tumor registry of atomic bomb survivors to show that such studies are possible using 275 hepatocellular carcinoma and 41 skin cancer cases. We used 23 relatively recent thyroid papillary carcinoma cases from persons living in the vicinity of the Chernobyl nuclear reactor accident for comparison. Degradation of DNA is severe in autopsy hepatocellular carcinoma samples but can be compensated for by decreasing the polymerase chain reaction product size. Increasing the amount of DNA that is used by a factor of 8 improved amplification efficiency from approximately 60 to 80%. Age of the samples was not as great a problem as was the source of procurement. The extracted DNA can be used for all types of assays that require polymerase chain reaction amplification, such as restriction fragment length polymorphism, single-strand conformation polymorphism, and direct sequencing. (Am J Pathol 1996, 149:399–406)

Polymerase chain reaction (PCR)-based procedures have revolutionized molecular biology. Nanogram quantities of tissue DNA permit study of a plethora of genes. Moreover, DNA from preserved tissues such as formalin-fixed and paraffin-embedded samples can be used as PCR templates.[1] The use of paraffin-embedded sections allows segregation of sites according to their pathologies. This is important in studies of mutations in genes associated with carcinogenesis because changes may be detected by comparing adjacent normal and tumor parts of the same tissue.[2,3]

In fields such as molecular oncology/epidemiology, reliance on archival tissues is often essential. In retrospective analyses of large populations, such as the atomic bomb survivors, the age of preserved tissues may date back over 40 years. Although paraffin-embedded tissues have been used since the turn of the century, five decades ago, the survivors' tissues were procured, fixed, and stored with no idea of their use a half-century later; certainly, use in PCR was not intended.

The conceivability of DNA analysis in 40-year-old specimens has been demonstrated using whole 5- to 10-μm-thick paraffin-embedded tissue sections.[4] And a number of studies address the usability of relatively new archival material for PCR analysis.[5,6] However, no reports exist of large studies using archival tissue material older than 20 years for PCR

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Table 1. *Tissue Procurement*

Procurement periods	No. of HCC samples	No. of skin samples	No. of TPC samples
1952–1960	6	4	0
1961–1965	29	4	0
1966–1970	49	2	0
1971–1975	46	6	0
1976–1980	42	15	0
1981–1985	81	7	0
1986–1989	22	3	0
>1989	0	0	23
Total	275	41	23

The HCC samples are from autopsies and the skin tumor and thyroid papillary carcinoma (TPC) samples from surgeries. The time between death and time of preservation of the liver tissues ranges from several hours to a day.

and subsequent molecular analyses of numerous gene sites. The tumor and tissue registries of atomic bomb survivors are a unique and extensive collection of such archival tissues. The potential for investigating various molecular aspects of radiation-related disease is great, but the feasibility for these studies or for any other similar retrospective molecular epidemiological study using decades-old (over 20 years) preserved tissues has never been documented.

In this report, we show the feasibility and limitations of an expansive molecular epidemiology study using archival materials collected from the atomic bomb survivors in comparison with recently procured samples.

Materials and Methods

Tissues

A total of 41 skin cancer cases (consisting of 27 basal cell carcinomas, 10 squamous cell carcinomas, and 4 Bowen's disease cases) and 275 hepatocellular carcinoma (HCC) cases of atomic bomb survivors ascertained from 1952 to 1989 were used for the present study. In addition, 23 cases of thyroid papillary carcinomas from children residing in the area contaminated by the Chernobyl accident of 1986 were also used. The skin and thyroid tumors are surgical specimens, and the HCC samples are autopsy specimens. The time between death and autopsy, when available, was obtained from medical records. All tissues were formalin fixed and paraffin embedded. See Table 1 for information on tissue procurement.

DNA Extraction

Two to five serial 5-μm-thick sections (9 to 100 mm^2) of each tissue were used for the study. The first section was stained with hematoxylin and eosin for histological assessment. This slide was also used as a guide to trace out regions of the section that were tumor for subsequent microdissection. Such steps are particularly important for enrichment of the tumor DNA, which is molecularly characterized in comparison to normal DNA. Additionally, microdissection allows individual molecular analyses of multiple tumor foci in a single section for studies in tumor progression, for example.

The remaining sections were scraped by scalpel into 1.5-ml conical centrifuge tubes, deparaffinized with 1 ml of xylene, washed with 100% ethanol, and treated in 100 μl of digestion buffer (50 mmol/L Tris-HCl, pH 8.5, 1 mmol/L EDTA, and 0.5% Tween 20) with 100 μg of proteinase K at 37°C for at least 48 hours. After phenol and chloroform extraction, genomic DNA was precipitated with ethanol.

Yield and purity of DNA were measured spectrophotometrically at 260 and 280 nm in all cases except the skin tumors. The skin sections were miniscule, averaging 25 mm^2 with the tumor often occupying less than 20% of the total area. The value and scarcity of the DNA precluded its use in measurement of quantity. When DNA yield was determined, all working solutions of DNA for PCR were adjusted to 50 ng/μl.

PCR Amplification

Various gene sites for subsequent use in restriction fragment length polymorphism, single-strand conformation polymorphism (SSCP), and direct sequencing analyses were amplified by PCR. The PCR products of various sizes were amplified from the p53, retinoblastoma (*Rb*), adenomatous polyposis coli (*APC*), and mutated in colon cancer (*MCC*) tumor suppressor genes and the *ras* family of oncogenes. The primers used are given in Table 2.

PCR amplification of 25 ng (unless otherwise stated) of template DNA was conducted in a 10-μl solution containing 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.4), 1.5 to 2.5 mmol/L $MgCl_2$, 200 to 600 μmol/L each dNTP, 2 pmol of PCR primers, and 0.5 U of *Taq* DNA polymerase (Perkin-Elmer, Cetus Corp., Emeryville, CA) under suitable thermal conditions. In general, the conditions were 35 cycles of 30 seconds at 94°C (denaturation), 1 minute at 60°C (annealing), and 1 minute at 72°C (elongation). In multiplex PCR, six pairs of primers were used instead of one pair. The resulting mixture of products after multiplex PCR was diluted by 100 and amplified by conventional PCR with each pair of primers separately. Products were visualized by ethidium bro-

Table 2. *List of Primers and Corresponding Gene Sites Used for Assessment of the Analyzability of Various Tissue Samples*

Primer set	Priming region	Amplicon size (bp)	Primer sequences*
1	p53 intron 1a	95	TTCCGCAGTTTCTTCCCATG TGTGTGTAAATGCCACCTCG
2	p53 intron 1b	158	TTTAGGAGTGGGGGTGGGAG GTAGAGTTGAGGAAAGTGCTGG
3	p53 codon 72	199	TTGCCGTCCCAAGCAATGGATGA TCTGGGAAGGGACAGAAGATGAC
4	p53 intron 6	150	TAGTGTGGTGGTGCCCTATG CATGGGGTTATAGGGAGGTC
5	p53 intron 7	164	TCCTCACCATCATCACACTG CCGCCGGAAATGTGATGAG
6	p53 3′ flanking reg	90	TCAGAAGGAAGTAGGAAGGACTCAG GAAGAGCCTCGGTTATGGGTATACA
7	p53 exon 5	151	TTCAACTCTGTCTCCTTCCT ACTGCTTGTAGATGGCCATG
8	p53 exon 5	216	TTCCTCTTCCTGCAGTACTC GCCCCAGCTGCTCACCATC
9	p53 exon 5	248	TTCAACTCTGTCTCCTTCCT CAGCCCTGTCGTCTCTCCAG
10	p53 exon 6	112	GCCTCTGATTCCTCACTGAT TGTCGAAAAGTGTTTCTGTC
11	p53 exon 6	223	TGGTTGCCCAGGGTCCCCAG GGAGGGCCACTGACAACCA
12	p53 exon 7	177	AGGCGCACTGGCCTCATCTT TGTGCAGGGTGGCAAGTGGC
13	p53 exon 7	237	CTTGCCACAGGTCTCCCCAA AGGGGTCAGCGGCAAGCAGA
14	p53 exon 8	165	CTATCCTGAGTAGTGGTAATC GTCCTGCTTGCTTACCTCGC
15	p53 exon 8	230	TTCCTTACTGCCTCTTGCTT AGGCATAACTGCACCCTTGG
16	*Rb* intron 1	195	CAGGACAGCGGCCCGGAG CTGCAGACGCTCCGCCGT
17	*Rb* intron 17	190	CTGCAGTCCCACCTCAGCCTCCTTAGTAGA GGATCCGCAGCTCTAGACTAATCCCAGCAC
18	*APC* exon 11	95	GGCCATTGCAGAATTATTGC AGCCATTCCAGCATATCGTC
19	*MCC* exon 15	80	TGATGCTGCCTCCCCATC TTGGTGAACTCCGCAGCC
20	H-*ras* codon 12	63	GACGGAATATAAGCTGGTGG TGGATGGTCAGCGCACTCTT
21	H-*ras* codon 61	75	AGACGTGCCTGTTGGACACATC CGCATGTACTGGTCCCGCAT
22	Ki-*ras* codon 12	107	GACTGAATATAAACTTGTGG CTATTGTTGGATCATATTCG
23	Ki-*ras* codon 61	106	AAGCAAGTAGTAATTGATGG CCTCCCCAGTCCTCATGTAC
24	N-*ras* codon 12	110	GACTGAGTACAAACTGGTGG CTCTATGGTGGGATCATATT
25	N-*ras* codon 61	103	GGTGAAACCTGTTTGTTGGA ATACACAGAGGAAGCCTTCG

Primer set 1 is from Ito et al[11]; set 2 is based on Willems et al[12]; set 3 is based on Ara et al[13]; set 4 is based on McDaniel et al[14]; set 5 is based on Prosser and Condie[15]; set 6 is based on Hoyheim et al[16]; primer sets 1 to 15 were determined using sequence data provided by Chumakov, Almazov, and Jenkins (GenBank accession number X54156, 1991); set 16 is from Bookstein et al[17]; sets 17 and 19 are from Greenwald et al[18]; set 18 is from sequence data provided by Kinzler et al[19]; sets 20 and 21 are from sequence data provided by Capon et al[20]; sets 22 and 23 are from sequence data provided by McGrath et al[21]; and sets 24 and 25 are from sequence data provided by Taparowski et al.[22]

*Of the primer pairs, the top is the upstream sequence and the bottom is the downstream sequence.

mide staining of 8% polyacrylamide gels after electrophoresis.

PCR amplification was scored positive when a band of appropriate size was able to be visualized and negative when no band could be seen. In negative cases, a second round of PCR using diluted (1:100) products of the first (negative) round as template was attempted. If still no band appeared, this procedure was repeated using 100 ng of the original DNA. If 100 ng were insufficient, 200 ng of the original DNA was used as a final attempt to amplify the DNA.

When PCR amplification was successful (positively scored), further amplification of the specific gene region was performed using diluted products of the first round of PCR so insufficiency for subsequent analyses was not a problem.

PCR-SSCP

The PCR products were diluted by 1:100 with deionized water, and 1 μl was used as the template for radioactive labeling by PCR in 10 μl of mixture containing 10 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 100 μg/ml gelatin, 10 μmol/L each dNTP, 0.2 μmol/L each of the primers, 1 μl of [α-^{32}P]dCTP (1.1×10^{11} Bq/ml), and 0.2 U of *Taq* DNA polymerase. After PCR amplification, the products were diluted 100-fold with 0.1% sodium dodecyl sulfate and 10 mmol/L EDTA followed by 1:1 dilution with a solution containing 95% formamide, 20 mmol/L EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol. After denaturation by heating at 95°C for 10 minutes, the samples were loaded onto an 8% polyacrylamide gel at 4°C. The gel was dried onto filter paper and autoradiographed.

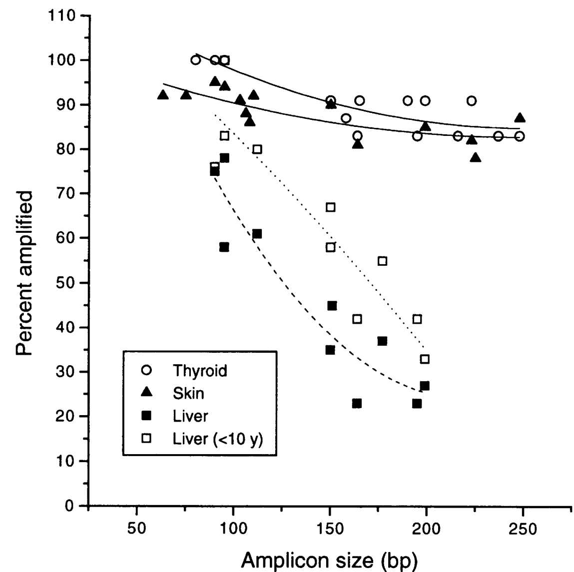

Figure 1. *Evidence for degradation of DNA in various tissues. The skin tumor samples were older than the thyroid tumor samples, but both were obtained during surgery. In contrast, the liver (HCC) samples were obtained from autopsy cases. The skin and liver were amplified first by multiplex PCR.*

Results

The percentage of cases that could be amplified and the amplicon size correlated well (polynomial fit with *t*-test for liver, $r = 0.94$, $P < 0.001$; skin, $r = 0.78$, $P < 0.001$; thyroid, $r = 0.88$, $P < 0.001$; see Figure 1), indicating that the efficiency of PCR amplification for a given amplicon size was not dependent on the gene site amplified in our system.

Extracted DNA from paraffin-embedded tissues appear degraded to various degrees. The skin and thyroid tumors demonstrated good efficiencies of PCR amplification within the size ranges most commonly used for our molecular analyses. Both skin and thyroid, which show similar amplification rates, although the skin tumors are older than the thyroid tumors, were obtained from surgery. However, the HCC samples, obtained through autopsy cases, showed considerable degradation as illustrated in Figure 1.

Additionally, when PCR amplification efficiencies of the liver cases procured from 1986 and later (that is, those that were less than 10 years old) were separately plotted *versus* amplicon size, there still was evidence of a substantial degree of degradation when compared with the skin and thyroid (Figure 1). However, there was a statistically significant ($P < 0.05$, using the Wilcoxon signed rank test) improvement in DNA integrity if the HCC tissues had spent less than 10 years in the paraffin. The improvement ranged from an increase in approximately 5 percentage points at the large amplicon sizes (approximately 200 bp) to 20 percentage points in the smaller amplicon sizes (approximately 100 bp). The median size of the HCC DNA fragments appears from the curves to be approximately 130 and 170 bp if the samples are less than 10 years old. Thus, the DNA of the HCC, whether more than or equal to 10 years old ("old") or less than 10 years old ("new"), are considerably more fragmented compared with new thyroid and old or new skin DNA.

The mean yields of DNA from the liver and thyroid tumors were 22.1 μg/case and 10.9 μg/case, respectively. And among the HCC samples, there was no dependence between age of the specimens and yield of DNA. As mentioned in Materials and Methods, the skin tumor sections were too small and therefore too precious to use for measurement of DNA, although roughly, assuming a proportional yield of DNA with the size of the section, less than 1 μg/case was probably extracted. Consequently, it is apparent that the difference in yield of DNA cannot be relied upon to explain the difference in efficiency of PCR amplification among the various tissue types.

Figure 2 shows the degradation of the HCC DNA according to the time between death and autopsy. Although degradation 2 hours postmortem shows considerable fragmentation, the overall shape of the

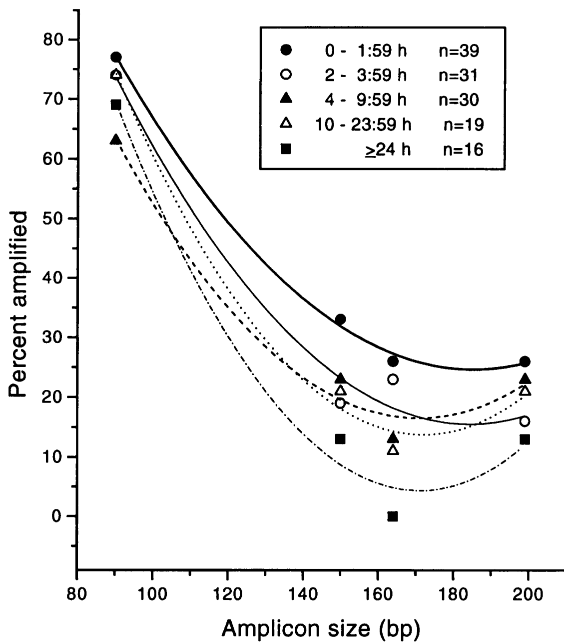

Figure 2. *Effect of postmortem elapsed time on the degradation of the liver (HCC) DNA. PCR was used to amplify 90-, 150-, 164-, and 199-bp sections of the 3'-flanking region, intron 6, intron 7, and exon 4 of p53, respectively. Elapsed time was calculated from time of death and time of autopsy recorded in the medical histories.*

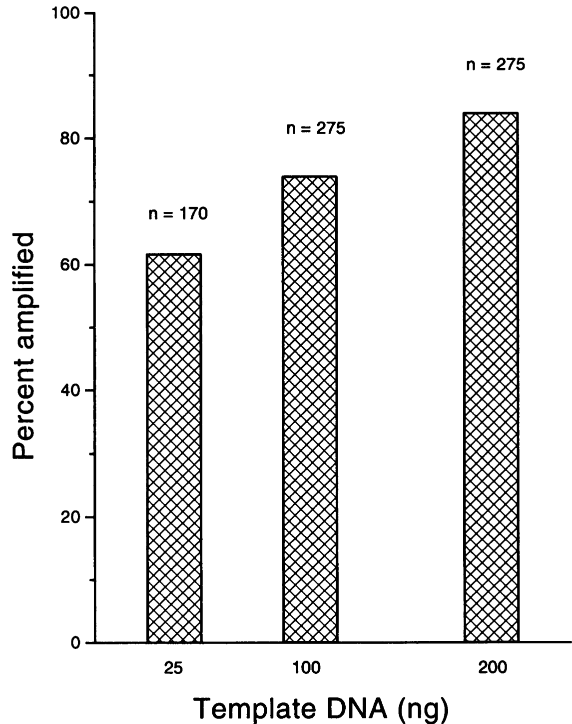

Figure 3. *Effect of template DNA quantity on the success of amplification. Liver (HCC) DNA was used as template for PCR amplification of a 110-bp section of N-ras (100- and 200-ng categories) or a 112-bp section of p53 exon 6 (25-ng category).*

degradation curves are quite similar; the median size of DNA fragments, as mentioned earlier, appears to be approximately 130 bp as approximated by the degradation curve. Thus, decreasing the amplicon size contributed considerably to the number of usable samples.

At a given amplicon size, the rate of successful PCR amplification can also be increased by using more template DNA, as shown in Figure 3. When the DNA quantity was quadrupled from 25 to 100 ng, there was an improvement from 62 to 74%, and use of 200 ng further improved the percentage of successful amplifications to 84%. In other words, approximately 25% of those HCC samples that could not be amplified with 25 ng of DNA were amplifiable when 100 ng were used, and among those remaining that could not amplified with 100 ng, 30% could be amplified when 200 ng were used.

With a decreased amplicon size, the proportion of samples that were successfully amplified from the oldest samples, which we anticipated would present the highest degree of degradation, was good, with little dependence on duration of preservation in paraffin, and thus permissive of molecular studies (Figure 4).

Liver DNA was multiplex PCR amplified using primers for sections of p53 exons 5, 6, 7, and 8. The subsequent PCR was conducted using each pair of primers separately using 1 μl of 1:100 diluted multiplex PCR product as template. The SSCP mutation analysis relies upon the tendency for single-stranded DNA to fold in a sequence-specific manner under nondenaturing conditions. Each different conformation travels at a different rate in polyacrylamide gel during electrophoresis. Therefore, a point mutation

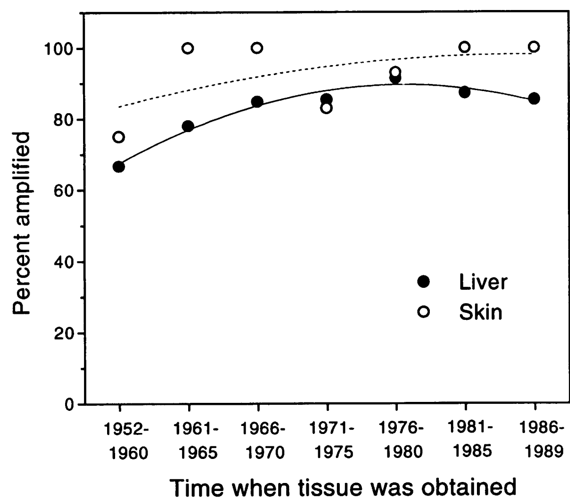

Figure 4. *Effect of age of the archival tissue on successful PCR amplification. Liver (HCC) or skin DNA (200 ng) was used as template for PCR amplification of a 110-bp section of N-ras. The number of cases per data point is given in Table 1.*

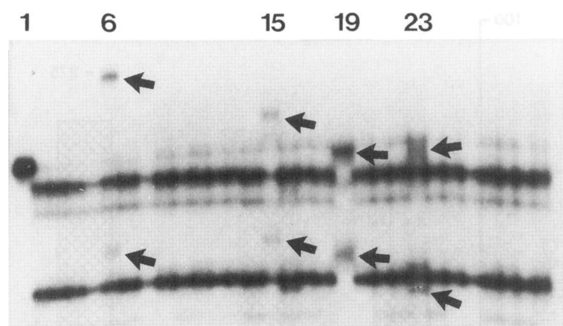

Figure 5. *A representative PCR-SSCP analysis of decades-old archival liver (HCC) DNA. The DNA was first amplified by multiplex PCR and then by single-primer-pair PCR. The figure shows one example using primers, in the second-step PCR, to amplify one-half of exon 5 (150 bp).* Lane 1 *contains double-stranded DNA. Mutant bands, designated by the* arrows, *are seen in* lanes 6, 15, 19, *and* 23.

will often (approximately 80 to 90% of the time) cause shifting of a band from the normal position. Figure 5 shows a representative SSCP analysis of p53 exon 5 of the liver DNA. Exon 5 is 186 bp such that a PCR product that includes the entire exon would be more than 200 bp; therefore it was amplified in two overlapping sections of approximately 150 bp each. After the DNA was amplified successfully, even if from the degraded liver DNA, the SSCP analysis was clearly adequate for mutation detection.

Discussion

Retrospective analyses using archival tissues are essential in molecular oncology/epidemiology. However, to obtain meaningful correlations and trends between clinical conditions and molecular events, a large number of tissue samples are necessary. In cases of rare diseases or diseases that develop over many years, prospective studies may be impractical. Formalin fixing and paraffin embedding of tissues allows not only the collection and storage of vast quantities of samples but also pathology-based investigations. Nevertheless, the feasibility of using archival tissue samples, some several decades old, in meaningful numbers for multiple-gene-site molecular analyses has never been reported. The accumulated epidemiological data showing an increased incidence of cancer among the atomic bomb survivors[7] suggests that the sizable collection of archival tissues from these survivors may be a valuable resource for elucidating the mechanisms of radiation carcinogenesis via molecular analysis. The work reported here clearly indicates that the use of these fixed and paraffin-embedded tissues is viable in molecular studies with the following caveats.

The time spent in paraffin appears to be a lesser confounding factor than the type of tissue, the method of tissue procurement, or time of fixation. Even HCC samples less than 10 years old showed considerable degradation, worse than skin tumor samples four times older.

The source of the tissues appears to play a large role in the degradation of DNA. The DNA from the liver samples was badly degraded and showed decreased amplification efficiency compared with the DNAs from the equally aged skin samples, which differed little from thyroid samples procured relatively recently. Why? The skin and thyroid tissues were obtained at surgery, whereas the liver tissues were obtained at autopsy, which undoubtedly provided time for the autolysis of the cells. Moreover, the liver, being a storehouse of degradative enzymes, may have contributed to the bad condition of the DNA. The integrity of DNA extracted postmortem is dependent on the organ of origin.[8,9] Fifteen-year-old or less paraffin-embedded spleen specimens, another tissue associated with various degradative enzyme activities, have been reported to show DNA degradation in contrast to freshly obtained tissues.[5] This comparison was done using a PCR amplicon size of approximately 1300 bp. Interestingly, at a smaller size (307 bp), the percentage amplified of these archival spleen samples was approximately 80%, similar to our results with the surgical skin and thyroid tumor samples. It may be inferred from the Discussion that the spleen tissues were obtained surgically.

Moreover, the fact that the amplification efficiency by PCR depended little on the length of time the tissues spent in the paraffin blocks suggests that the most important factor is the time before a sample is embedded. The amplification efficiency of the liver samples improved with a decrease in the time between death and autopsy but, interestingly, not as much as expected. For example, at a 199-bp amplicon size, tissues procured more than 24 hours postmortem showed 13% amplification, whereas the tissues procured less than 2 hours postmortem showed only a 26% amplification. Postmortem degradation may be rapid, especially in large, deep-seated organs like the liver where temperatures may remain at near physiological levels for an extended period of time, as compared with surgically removed skin samples that are small and can quickly be cooled.

Certainly the differences in successful amplifications among tissue types or between surgically obtained and autopsy-obtained specimens is not attributable to the total DNA yield. If such were the case,

the skin tumor DNA, which had the smallest yield, should have showed the worst amplification efficiency.

Another factor that ought to be considered is the time of fixation in formalin before paraffin embedding. Long preservation in formalin greatly diminishes amplification efficiency as does the type of fixative.[10] Although, unfortunately, the times of fixation of the samples are not available from the medical records, it is unlikely that the liver (autopsy) samples spent more time in the fixative than the skin (surgery) samples. In postmortem examinations, tissues are immersed in fixative usually during or just after the gross examination of organs. The time consumption for preparation of microscope tissue slides is not very much different from that in surgical pathology. However, it should be noted that autopsy liver is also preserved as a wet organ or tissue, being immersed in fixative for years. In such cases, even if the primary fixation were adequately done, tissue quality suffers from chemical changes that occur, including formic acid formation, during the longstanding organ storage.

However, reducing the size of the PCR products can effectively increase the number of usable samples in cases in which the DNA is badly degraded. In addition, increasing the amount of template DNA can slightly increase the percentage of successful amplifications. In some cases this may be preferable. For example, when a predetermined sequence is to be amplified for SSCP or direct sequencing, reduction of amplicon size will necessarily increase the number of PCR amplifications to accommodate the full sequence of interest; this may not be desirable if there are many cases to be analyzed. For restriction fragment length polymorphism analysis, there is no problem as long as the polymorphic site is within the section of the gene to be amplified. Also, in many cases, the size of the available tissue is miniscule and the small amount of available DNA may force the use of smaller amplicon size.

If several gene sites are to be analyzed, multiplex PCR may be successfully utilized on archival samples when DNA quantities are limited. Thus, for example, 25 ng of template DNA would suffice when conventionally 150 ng would be required to analyze six gene loci. The template used for the SSCP analysis shown in Figure 4 was a diluted aliquot of a multiplex PCR product solution containing amplified sections of p53 exons 5, 6, 7, and 8. The samples showing mutant bands were subsequently successfully sequenced for confirmation and further analysis (data not shown).

We have demonstrated the feasibility of using decades-old archival tissues for use in large-scale molecular oncological/epidemiological studies. Such investigations can help to elucidate the molecular mechanisms of many clinical conditions in which prospective studies are impractical due to the extended period of time required for the genesis of the disease or impossible due to the uniqueness of the study cohort as in the case of the atomic bomb survivors.

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