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Original Article

Improved PCR amplification for molecular analysis using DNA from long-term preserved formalin-fixed, paraffin-embedded lung cancer tissue specimens

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Abstract: Archival tissue specimens are valuable resources of materials for molecular biological analyses in retrospective studies, especially for rare diseases or those associated with exposure to uncommon environmental events. Although successful amplification with PCR is essential for analysis of DNA extracted from archival formalin-fixed, paraffin-embedded (FFPE) tissue specimens, we have often encountered problems with poor PCR amplification of target fragments. To overcome this, we examined whether heat treatment in alkaline solution could efficiently restore the PCR template activity of DNA that had already been extracted from FFPE lung cancer tissue specimens. The effect of the heat treatment was assessed by PCR for the *TP53* gene and other lung cancer-related gene loci. The heat treatment of DNA samples in borate buffer resulted in successful PCR amplification of DNA fragments ranging from 91 to 152 bp. This technique for restoration of template activity of DNA for PCR amplification is very simple and economical, and requires no special apparatus, so it may be applicable for molecular analysis of DNA samples from FFPE tissue specimens at various laboratories.

Keywords: FFPE, lung tissue specimen, DNA restoration, PCR

Introduction

Radiation Effects Research Foundation (RERF) has collected and stored a large number of archival formalin-fixed, paraffin-embedded (FFPE) tissue specimens obtained from atomic bomb (A-bomb) survivors. Archival tissue specimens have become valuable, especially for histological and molecular biological analyses of radiation-associated cancers in the case of RERF. Most of these specimens were fixed with formalin, embedded in paraffin and stored at room temperature, and DNA extracted from such specimens has been used as templates for PCR amplification. However, we have often had problems with poor PCR amplification, i.e., insufficient amounts of DNA functioned as template compared with intact DNA, and the limitations in amplifiable DNA size [1]. These issues can be attributable at least in part to DNA fragmentation and chemical modifications caused by fixation in formalin [2].

Recently, improvements of DNA isolation from FFPE tissue specimens were reported on the basis of the antigen retrieval principle [3, 4]. FFPE tissue specimens, without deparaffinization, were incubated in alkaline buffer at high temperatures (80-120°C) for 20 min, followed by phenol/chloroform treatment and ethanol precipitation, which resulted in higher yields and better quality of DNA in terms of PCR amplification. The key mechanism of action of heating in alkaline solution for DNA may be to denature and hydrolyze proteins, resulting in 1) rupture of cell and nuclear membranes and 2) breakage of cross-linkages caused by formalin fixation [3].

It was also reported that the chemical modification of bases of RNA by fixation in formalin can

Table 1. List of primers and PCR conditions

Gene or marker	Region	Primer sequence (5'- -3')	Annealing temperature (°C)	Mg^{2+} concentration (mM)	Extension temperature (°C)	Primer concentration (nM)	Size (bp)
TP53 (exon 8)	17p13	TGAGTAGTGGTA-ATCTACTGG	55	3 as $MgCl_2$	72	200	152
		TTGCTTACCTC-GCTTAGTGC					
TP53 (exon 7)	17p13	AGGTTGGCTCT-GACTGTACC	55	3 as $MgCl_2$	72	200	120
		CTCCTGACCTG-GAGTCTTCC					
D3S1266 (for *RARB*)	3p22-24	ACCTT-TATGGGAGT-GTCTTTGGGAGA	60	4 as $MgSO_4$	68	250	112
		TGGATG-GAAAANACGTAT-GTGTCTGTG					
D3S4614/Luca8.2 (for *RASSF1*)	3p21.3	GCTGAGAAATCT-CAATTGTGGGTG	58	3 as $MgCl_2$	72	500	126
		GGCTGCTGAG-CAGTGTCAGAC					
D3S4103 (for *FHIT*)	3p14.2	GCAGAG-CAAGACCCTATCT-CAT	60	2.5 as $MgSO_4$	68	500	91
		TGCCTTGGGTAG-ATTTATACCTG					
D9S171 (for *CDKN2A*)	9p21	CTCATCTCTGTCT-GCTGCCTCCT	59	3.5 as $MgSO_4$	68	250	109
		TTCTTGGGGC-TACTTTATTAA-CAATCA					

be partially removed by incubation in TE buffer (pH 7.0) [5] or citrate buffer (pH 4.0) [6] at 70°C, resulting in restoration of template activity for RT-PCR. Based on the notion that heat treatment in alkaline buffer can be usable for DNA already extracted from FFPE tissue specimens in order to restore template activity for PCR, we examined whether heat treatment of DNA which had already been extracted from FFPE lung cancer tissue specimens in alkaline solution could efficiently restore PCR amplification.

Materials and methods

Tissue specimens and DNA extraction

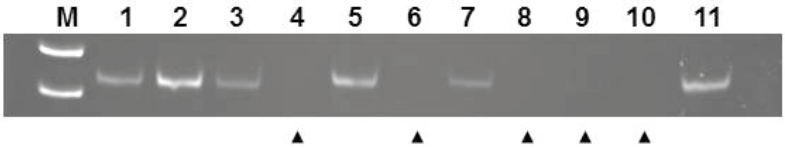
Subjects of this study comprised 11 lung cancer cases found in a Life Span Study cohort of A-bomb survivors. FFPE lung cancer tissue specimens from subjects who had undergone surgery at local hospitals in Hiroshima during 1987 - 2001 were obtained and unlinkably anonymized through the Study Group on Atomic Bomb Diseases, entrusted by the Ministry of Health, Labor and Welfare (MHLW) of Japan [7]. After deparaffinization of 5 μm sections using Hemo-De (Falma, Tokyo, Japan), the sections were stained with methylgreen (Sigma-Aldrich, St. Louis, MO) and dissected manually or by laser microdissection system AS LMD (Leica, Wetzler, Germany). DNA was extracted from microdissected tissue sections using QIAamp DNA Micro kit (QIAGEN, Hilden, Germany), according to the manufacturer's instruction.

This study was conducted under approval of the Genetic and Medical Ethics Commission at Hiroshima University, and the Human Investigation Committee and the Ethics Committee for Genome Research at RERF.

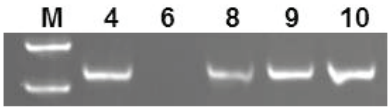
Heat treatment of DNA in borate buffer and PCR

Four hundred ng of DNA was heated in 100 μl of 25 mM citrate-acetate buffer (pH 5.0 or 6.0), 25 mM Tris-HCl buffer (pH 7.0 or 8.0) or 25 mM borate-NaOH buffer (pH 9.0, 10.0, or 11.0) at various temperatures (25-120°C) for 30 min, followed by ethanol precipitation in the pres-

A Before DNA restoration



B After DNA restoration



C

Case #	Duration of storage (yrs)	PCR for					
		TP53 (exon 8)	*TP53* (exon 7)	D3S1266	D3S4614	D3S4103	D9S171
1*	14						
2*	15						
3*	18						
4	18						
5	17						
6	15						
7*	13						
8	12						
9	7						
10	4						
11*	4						

Figure 1. Improved PCR amplification by heating archival FFPE lung cancer tissue DNA in borate buffer. A: Polyacrylamide gel electrophoresis of PCR products (152 bp) for *TP53* (exon 8) using 10 ng of non-preheated DNA. Lane M is molecular size marker. Lanes 1-11 indicate PCR products of each lung cancer case. Closed arrow heads indicate cases with non-amplified DNA. B: PCR products amplified with 10 ng of preheated DNA. C: Summary of PCR amplification for *TP53* and lung cancer associated gene loci using non-preheated and preheated DNA. Empty grid indicates successful PCR using non-preheated DNA (in this case, PCR amplification using preheated DNA was not performed). Gray grid indicates successful PCR amplification using only preheated DNA. Black grid indicates no PCR amplification using either non-preheated or preheated DNAs. Cases with asterisks indicate successful PCR amplifications of all target fragments using 10 ng of non-preheated DNA.

ence of Ethachinmate (Nippon Gene, Tokyo, Japan) as carrier. Then, DNA was dissolved in TE buffer. As for PCR template, 10 ng of non-preheated or preheated DNA was used. PCR was performed using 0.5 U of Expand High Fidelity enzyme mixture (Roche Diagnostics, Basel, Switzerland) for *TP53*, 1 U of Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) for D3S4614/Luca8.2, and 1 U of Platinum *Taq* DNA polymerase High Fidelity (Invitrogen) for D3S1266, D3S4103, and D9S171 in volume of 20 µl containing 1 x PCR buffer, 200-400 µM each of deoxyribonucleotide triphosphate mixture, Mg^{2+}, and each primer. PCR conditions consisted of initial denaturation (95°C for 2-3 min), followed by 44 cycles (40 cycles for *TP53*) of denaturation at 94°C for 30 sec, annealing for 1 min, extension for 30-60 sec, and a final extension for 7 min. Primer sets, temperatures of annealing and extension, and concentrations of Mg^{2+} are summarized in **Table 1**.

Results and discussion

Degraded DNA prepared from a colorectal cancer cell line, Colo210, that had been fixed with 15% unbuffered formalin for 3 days prior to DNA preparation, was used to preliminarily determine optimal conditions for heat treatment of DNA. As a result, preheating DNA to

100 or 120°C in buffer with pH 11 was most effective for PCR amplification (data not shown). To assess the effect of preheating DNA extracted from FFPE lung cancer tissue specimens on PCR amplification, DNA was incubated in borate buffer (pH 11.0) at 100°C for 30 min. PCR amplification of exon 8 in the *TP53* gene with 10 ng of preheated DNA indicated that the treatment had restored DNA template activity for PCR amplification in 4 of 5 samples (**Figure 1A** and **1B**). Similar effects were observed in PCR amplification of several lung cancer-associated gene loci, summarized in **Figure 1C**. However, one case (#6) showed no effects of heat treatment on most of its PCR amplifications, possibly because conditions for fixation and/or storage of the tissue specimen were so severe that preheat treatment could not restore template activity of DNA. Furthermore, in two FFPE thyroid tissue specimens stored at RERF, PCR amplification of *TP53* with only 2.5 ng of preheated DNA produced DNA fragments longer than 200 bp, while 25 ng of non-preheated DNA was required for the same PCR amplification (data not shown).

These observations suggest that heat-treatment either during DNA extraction or after DNA extraction can restore DNA template activity for PCR amplification, although the extent of restoration depends on conditions of fixation and storage of tissue specimens. This improvement of PCR amplification may be due to the partial elimination of chemical modification of nucleic acids generated by fixation with formalin, as seen in heat-treatment of RNA from FFPE tissue specimens [5, 6].

This technique is very simple, cost-saving, and requires no special apparatus. We think it will be applicable for various molecular analyses of DNA samples from long-term preserved FFPE tissue specimens in general.

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Conflict of interest statement

The authors have no conflicts of interest to disclose.

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References

[1] Iwamoto KS, Mizuno T, Ito T, Akiyama M, Takeichi N, Mabuchi K and Seyama T. Feasibility of using decades-old archival tissues in molecular oncology/epidemiology. Am J Pathol 1996; 149: 399-406.

[2] Srinivasan M, Sedmak D and Jewell S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol 2002; 161: 1961-1971.

[3] Shi SR, Cote RJ, Wu L, Liu C, Datar R, Shi Y, Liu D, Lim H and Taylor CR. DNA extraction from archival formalin-fixed, paraffin-embedded tissue sections based on the antigen retrieval principle: heating under the influence of pH. J Histochem Cytochem 2002; 50: 1005-1011.

[4] Shi SR, Datar R, Liu C, Wu L, Zhang Z, Cote RJ and Taylor CR. DNA extraction from archival formalin-fixed, paraffin-embedded tissues: heat-induced retrieval in alkaline solution. Histochem Cell Biol 2004; 122: 211-218.

[5] Masuda N, Ohnishi T, Kawamoto S, Monden M and Okubo K. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. Nucleic Acids Res 1999; 27: 4436-4443.

[6] Hamatani K, Eguchi H, Takahashi K, Koyama K, Mukai M, Ito R, Taga M, Yasui W and Nakachi K. Improved RT-PCR amplification for molecular analyses with long-term preserved formalin-fixed, paraffin-embedded tissue specimens. J Histochem Cytochem 2006; 54: 773-780.

[7] Yasui W and Oue N. Systematic collection of tissue specimens and molecular pathological analysis of newly diagnosed solid cancers among atomic bomb survivors. In: Shibata Y, Namba H, Suzuki K, Tomonaga M, editors. Radiation Risk Perspectives. Amsterdam: Elsevier. 2007. pp: 81-86.