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# Randomized Terminal Linker-dependent PCR: A Versatile and Sensitive Method for Detection of DNA Damage

ZHANG ZHI-WEI AND HENG ZHENG-CHANG

*Department of Environmental Health, School of Public Health, West China Center of Medical Sciences, Sichuan University, Chengdu 610041, China*

**Objective** To design and develop a novel, sensitive and versatile method for *in vivo* foot printing and studies of DNA damage, such as DNA adducts and strand breaks. **Methods** Starting with mammalian genomic DNA, single-stranded products were made by repeated primer extension, these products were ligated to a double-stranded linker having a randomized 3′ overhang, and used for PCR. DNA breaks in *p53* gene produced by restriction endonuclease *Afa*I were detected by using this new method followed by Southern hybridization with DIG-labeled probe. **Results** This randomized terminal linker-dependent PCR (RDPCR) method could generate band signals many-fold stronger than conventional ligation-mediated PCR (LMPCR), and it was more rapid, convenient and accurate than the terminal transferase-dependent PCR (TDPCR). **Conclusion** DNA strand breakage can be detected sensitively in the gene level by RDPCR. Any lesion that blocks primer extension should be detectable.

**Key words:** Randomized terminal linker-dependent PCR; LMPCR; *p53* gene; DNA damage

## INTRODUCTION

Only a few methods are available for quantitatively detecting DNA lesions in specific genes of the mammalian cells. One such method is ligation-mediated PCR (LMPCR)[1, 2]. LMPCR has been used successfully in numerous *in vivo* studies of the mammalian cells for detection of DNA damage, including DNA strand breaks[3], DNA adducts[4, 5], and oxidative DNA damage[6]. In the conventional method of LMPCR, the substrate genomic DNA is cut at the sites of altered bases either with a specific enzyme or by use of chemical reagents. The resulting single-strand breaks are converted to blunt-ended termini by extension from a gene-specific primer and are ligated to a double-stranded linker. The sequences between the linker and a second (nested) gene-specific primer are amplified by PCR and the products are visualized.

LMPCR is very sensitive but has some limitations[7]. For example, it measures directly only nicks or breaks in DNA and requires ligatable 5′ ends. The single strand ligation PCR (SLPCR) can eliminate the limitation by repeated primer extension and single strand ligation[8], but its ligation efficiency is very poor[7]. The terminal transferase-dependent PCR (TDPCR) method depends on cohesive-end ligation to the 3′ ends of DNA molecules resulting from primer extension, followed by ribonucleotide tailing by terminal deoxynucleotidyl transferase (TdT). TDPCR has a high sensitivity but in most cases DNA damage cannot be mapped as

Biographical note of the first author: Zhang Zhi-Wei, male, born in June, 1974, Ph.D candidate, majoring in molecular genetic toxicology. E-mail: zzwwcums@263.net

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precisely as with LMPCR[5].

The randomized terminal linker-dependent PCR (RDPCR) method reported here (Fig. 1) provides an alternative to the techniques mentioned above. A randomized cohesive-end linker is ligated directly to the 3′ ends of DNA molecules resulting from primer extension. RDPCR does not require ribonucleotide tailing and thus can be more quickly, convenient and accurate than TDPCR. At the same time the method should enable use of DNA-damaging agents whose products on DNA cannot easily be converted to ligatable 5′ termini. The DNA lesion model was produced by restriction endonuclease *Afa*I and the strand break of *p*53 gene would be detected using RDPCR.

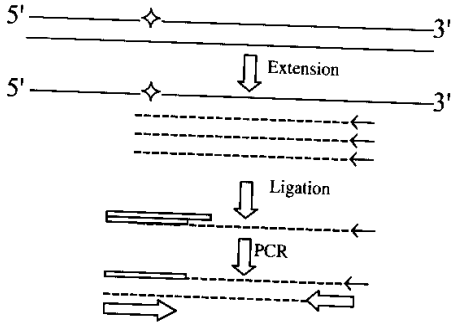


FIG. 1. Schematic outline of RDPCR. A DNA lesion in the starting DNA is indicated by a small star.

## MATERIALS AND METHODS

### *Cells and DNA Preparation*

Culture of the TK6 cells, DNA isolation, and cleavage of genomic DNA by *Afa*I were performed as described[9].

### *Primers and Linkers*

All oligonucleotides were synthesized or end-modified by Shanghai Sangon Ltd. Both the gene-specific primers P1, P2, P3 and P3′ for the exon 7 of *p*53 gene, and the linker, linker-primer used for LMPCR were the same as described previously[9].

Linkers for RDPCR were as follows:

Upper oligonucleotide: 5′-GCGGGTGACCCGGAGATCTGAATTCNNN-3′,with or without an amine blocking group at their 3′ termini. N stands for any one of A, T, G, and C.

Lower oligonucleotide: 5′-GAATTCAGATCTCCGGGTCACCCGC-3′. The 5′ terminus was phosphorylated.

Linker annealling was performed by incubation in a 100 μL reaction solution consisting of 20 μmol/L upper and lower oligonucleotides, and 250 mmol/L Tris-HCl, pH 7.7. The mixture was heated to 95℃ and allowed to cool gradually.

Linkers-primer for RDPCR: 5′-GCGGGTGACCCGGAGATCTGAATTC-3′.

*Probe*

The probe was made as described[9]. In brief, the exon 7 of *p*53 gene was amplified by PCR using the primers P3 and P3′. After agarose gel electrophoresis, the 186bp object band was cut down and the PCR product was got back. A same new PCR reaction was performed except by use of the purified exon 7 as template, and employed dNTP containing DIG-labeled dUTP (Roch). A portion of the amplified product was electrophoresed, and the rest was precipitated with ethanol. The probe was quantified according to the instruction manual of DIG DNA labeling and detection kit (Roch).

*RDPCR and LMPCR*

During the set-up of RDPCR reactions and between the heating steps, manipulations were performed on ice unless stated otherwise. 0.5 μg genomic DNA digested by *Afa*I was linearly amplified in a 25 μL reaction solution consisting of 1 U of Vent (exo-) DNA polymerase (New England Biolabs, NEB), 1×ThermoPol Buffer (NEB), extra 2 mmol/L $MgSO_4$, 200 μmol/L each dNTP, and 80 nmol/L primer P1. The temperature cycles were 1 min at 95℃ (5 min at 95℃ for the first cycle), 2 min at 56℃, and 1 min 20 s at 75℃. After 30 thermal cycles, each sample was transferred into another tube containing 75 μL of a solution composed of 0.4 mol/L sodium acetate, pH 5.2, 2.5 mmol/L EDTA and 1 μg tRNA, and was precipitated with 250 μL of ethanol. The precipitate was dissolved in 30 μL of sterile $H_2O$. After the addition of 4 μL of 10×ligase buffer solution, 5 μL of 20 μmol/L linker and 1 μL of T4 DNA ligase (NEB,400 U/μL), the mixture was incubated at 17℃ overnight. After ethanol precipitation, PCR was done in a 50 μL reaction consisting of the ligation product, 2 U of DeepVent DNA polymerase (NEB), 1×ThermoPol Buffer (NEB), extra 2 mmol/L $MgSO_4$, 200 μmol/L each dNTP, and 200 nmol/L primer P2 and linker primer. PCR was done using 30 cycles of 1 min at 95℃ (5 min at 95℃ for the first cycle), 2 min at 67℃ and 1 min at 75℃. After thermal cycling, the sample was precipitated with ethanol.

LMPCR was conducted exactly as described[9].

*Southern Hybridization*

One-third of each RDPCR or LMPCR product was analyzed by electrophoresis on 24 g/L agarose gels and was vacuum transferred to positive charged nylon membranes. Hybridization and colorsubstrate were carried out according to the instruction manual of DIG DNA labeling and detection kit (Roch).

## RESULTS

Fig.1 shows schematically RDPCR procedures. The RDPCR procedure begins with repeated primer extensions, producing multiple copies of the template strands. To enable PCR amplification of the newly synthesized, single-stranded products, a linker must be ligated to the 3′ ends. To accomplish this, a double-stranded DNA linker with a randomized 3′ overhang of three bases was used. It should be noted that in RDPCR only the lower, newly-synthesized strands participate in the PCR step, while in LMPCR only the upper, old template strands participate.

The results of RDPCR are shown in Fig.2. There were 146 bases from the only cutpoint

of *Afa*I in exon 7 of *p*53 gene to the 5′ end of the primer P2, so a 171bp segment should be made by RDPCR because the linker primer had 25 bases. Lane 6 was the purified 186bp exon 7 of *p*53 gene, which was used as positive control and marker here. Both lane 3 and lane 5 showed the results of RDPCR. The linker used in lane 5 had an amine blocking group at their 3′ termini in its upper strand but the one used in lane 3 had not. The signal of lane 3 was very poor, while the band of lane 5 was clear and strong. Lanes 1, 2 and 4 were the negative controls, and no signals were detected.

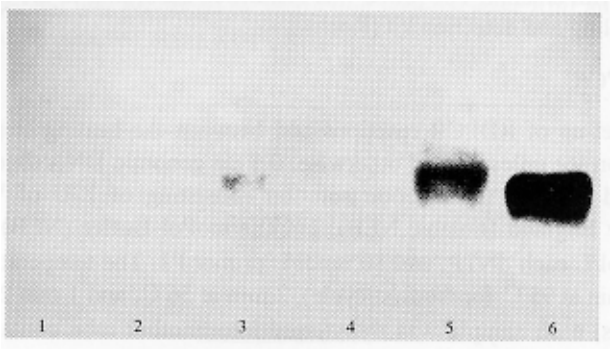


FIG.2. The results of RDPCR. Both lane 3 (its linker without an amine blocking group) and lane 5 (with an amine group) show the results of RDPCR.; Lane 1: genomic DNA; Lane 2: genomic DNA cut by *Afa*I; Lane 4: PCR amplification of genomic DNA using primer P2 and the linker primer; Lane 6 : purified 186bp exon 7 of *p53* gene.

As Fig. 3 shows, data from RDPCR and LMPCR were in parallel, with the same amount of starting materials in each reaction. DNA isolated from TK6 cells and subjected to *Afa*I cleavage was served as the substrate. The breaks of *p*53 gene in the same position could be visualized by LMPCR (lane 1) and RDPCR (lane 4). To quantitatively measure signals and compare RDPCR with LMPCR, the intensity of bands in both LMPCR and RDPCR was measured by use of UVIband V.97 Software (UVItec, UK). The intensity values were 90 750 and 635 654 respectively. The signal intensity by RDPCR was 7 times of that by the conventional LMPCR. With TDPCR, however, the background also increased somewhat.

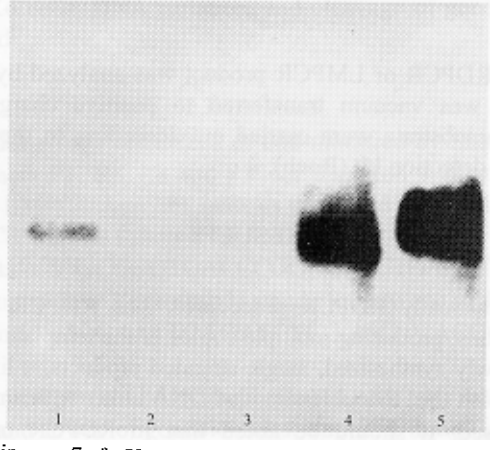


FIG.3.Detection of DNA damage in exon 7 of *p*53 gene by conventional LMPCR or by RDPCR. Lane 1: LMPCR; Lane 4: RDPCR; Lane 5: 186bp positive control; Lane 2 and Lane 3: negative controls.

## DISCUSSION

DNA damage is an early detectable key step during the environmental carcinogensis. There are plenty of methods which can be applied to research on DNA damage. Among them, LMPCR is extraordinary because of its sensitivity, and LMPCR can also map DNA damage of specific genes at single-nucleotide resolution. Some important results have been obtained using LMPCR. For example, Tornaletti *et al.*[10, 11] confirmed that the distribution of UV photoproducts along *p*53 gene had direct relation to mutations in skin cancer, and repair of the damages at those hotspots was slower than at others. Denissenko *et al.*[12, 13] also discovered that benzo(a)pyrene adducts preferred to format and repair slowly at lung cancer mutational hotspots in *p*53 gene. The results from LMPCR showed that DNA damage-repair might be related to cancers directly.

However, LMPCR requires DNA single-strand breaks with 5′-phosphates for ligation. Only a few DNA-damaging agents, such as bleomycin[14], make this type of terminus. For DNA adduct detection, the adduct must be converted to strand breaks firstly by a specific enzyme or chemical reagent, but these reagents require a special source to which many may not have access. Many DNA-damaging agents have not been used by LMPCR because of the absence of adequate cleavage methods.

Fortunately, LMPCR has been improved and extended in several ways. Grimaldi *et al.*[8]successfully combined linear and exponential amplifications by using T4 RNA ligase to join a single-stranded linker to the single-stranded products of the primer extension. This is a useful procedure, and some important results were obtained for cisplatin adducts. However, DNA is not a preferred substrate of RNA ligase, the ligation of single-stranded DNA molecules is a slow and inefficient reaction. To circumvent this difficulty, Komura modified the 3′ termini of the primer extension products by ribo-tailing with TdT, so that the homopolymeric tails could be efficiently ligated to a double-stranded linker by the cohesive-end ligation using T4 DNA ligase. TDPCR produces stronger signals than LMPCR, but the number of ribo-tailing is not changeless.

As compared with TDPCR, RDPCR also depends on the cohesive-end ligation by T4 DNA ligase. Therefore, RDPCR should possess high sensitivity, too. In this study, the signal intensity by RDPCR was seven times of that by conventional LMPCR. The results showed a surprising consistence with that of TDPCR by 30 cycles of first primer extension[7]. But RDPCR employs a linker with a randomized 3′ overhang so the step of ribo-tailing in TDPCR is bypassed and the deficiency of TDPCR could be overcome. Because of the use of the randomized 3′ cohesive-end, any primer extension product can be ligated effectively, which may increase the sensitivity of RDPCR, but some non-specific ligation reactions may be done if the reaction condition was not optimized. The decrease of cycle number of the first primer extension should be helpful in reducing the background. In order to prevent self-ligation, the linkers used in RDPCR should be blocked either by means of ddNTP or with a blocking group at the 3′ termini of the upper strand.

RDPCR takes the advantage of the fact that DNA lesions can stop polymerase *in vitro*[15]. Hence RDPCR should be useful in detecting many kinds of damage only if the damage stops polymerase. It has been noted that highly-mutagenic adducts stop DNA polymerase more strictly than lessly-mutagenic adducts[16], so polymerase stop assay may preferentially detect the lesions with the most biological significance. At the same time, RDPCR should be applied to detect DNA damage produced by environmental pollutants whose ingredients are unknown. In addition to the combination of Maxam-Gilbert cleavage and sequencing gel electrophoresis, RDPCR should be able to detect DNA damage at single-nucleotide resolution.

To sum up, the ingenuity makes RDPCR a versatile and sensitive method for the detection of DNA damage. Although much remains to be further studied for RDPCR, we are

optimistical about its application.

## ACKNOWLEDGEMENT

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