Semiquantitative Chemiluminescent Detection of UV-B-Induced Point Mutations in the *p53* Tumor-Suppressor Gene

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ABSTRACT

The technique of allele-specific PCR (AS-PCR) enables the detection of a small number of mutant alleles in a large number of wild-type (WT) alleles. We used the AS-PCR technique and Southern blotting, using a nonradioactive labeled probe to analyze the formation of point mutations in the tumor-suppressor gene p53 of primary keratinocytes after UV-B irradiation. These permanent mutations resulting from CC dimers occur at distinct "hot-spots", one of which is affected in the human keratinocyte cell line HaCaT. This enabled us to establish the method with a defined positive control template, which also allowed semiquantitative determination of the mutation frequency. This, and the determination of the detection limit, was done with the use of serial dilutions of WT genomic DNA from primary keratinocytes with mutant genomic HaCaT DNA in the AS-PCR assay.

Nonradioactive methods have many advantages over their radioactive counterparts, including increased safety and the use of labeled probes that are stable over a long period of time. One particular example in which a sensitive technique is required is the detection of mutations in cultured cells. In contrast to homogeneously mutated populations of cells, such as in tumor tissue, mutations in cultured cells require a sensitive detection method because they occur infrequently, and only a relatively few cells are affected. Also, the method for detecting genomic mutants has to distinguish between mutated and wild-type (WT) alleles. The principle of allele-specific polymerase chain reaction (AS-PCR) is based on a 3' OH mismatch of one primer with the WT DNA (5,7). The primer can hybridize with the WT template strand, but it is elongated less efficiently by several orders of magnitude compared with a perfectly matched primer on mutant DNA and depending on the bases involved (5,8). This results in poor amplification of WT alleles, whereas the mutant alleles form a visible PCR product. The aim of our study is to detect a CC→TT tandem transition in the tumor-suppressor gene *p53*, codons 281/282 on exon 8. Point mutations in the *p53* gene were demonstrated in many tumors (9). The UV-B-induced CC→TT tandem transitions might play an important role, especially in skin cancer development

(3). These mutations at dipyrimidine sites are known to be specifically formed following UV-B irradiation (2). The *p53* gene has several "hot spots" (4) in which the CC→TT mutations occur at certain codons, including codons 281/282. In addition, the spontaneously immortalized keratinocyte cell line HaCaT (1) includes the same mutation (6) and, thus, can serve as a positive control. We show that the use of serial dilutions of mutant DNA makes a semiquantitative AS-PCR possible. This allows determination of the detection limit and the generated mutation frequency in UV-B-treated cultured cells.

Neonatal human foreskin keratinocytes were grown in Keratinocyte Growth Medium plus 0.15 mM Ca⁺⁺ (Clonetics, San Diego, CA, USA) to 40% confluence and irradiated with 7.3 mJ/cm² UV-B. The cells were subcultured for three weeks to allow repair of transient mutations and to establish the mutations in the genome by several cell divisions. DNA was then isolated from the irradiated cells and unirradiated control keratinocytes with the QIA-amp[®] Blood and Tissue DNA Isolation Kits (Qiagen GmbH, Hilden, Germany) and also isolated from the HaCaT cell line. Nested PCR was performed on the genomic DNA. In an initial PCR, a 782-bp fragment, encoding the entire *p53* exons 7 and 8, was amplified by using 10⁵ copies of genomic DNA (keratinocyte and HaCaT) as the template.

The reaction volume of 50 μL contained 1 U *Taq* DNA Polymerase, 10 $\times$ PCR buffer, 1.5 mM magnesium chloride (all from Boehringer Mannheim, Mannheim, Germany), 200 μM each dNTP and 0.4 μM forward primer, 5'-GTG TTA TCT CCT AGG TTG GCT CT-3', and reverse primer, 5'-CAA GAC TTA GTA CCT GAA GGG TG-3'. The amplification program used was: 96°C for 2 min, 30 $\times$ (55°C for 1 min, 72°C for 1 min and 94°C for 30 s) and 72°C for 20 min in a PTC-200TM Thermal Cycler (MJ Research, Watertown, MA, USA). The products of the reactions were purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim) and eluted in water. The second AS-PCR was performed on 250 ng of the purified *p53* exon 7 plus eight PCR products. The reactions were carried out in 100- μL vol

using 1 U *Taq* DNA polymerase, 10 $\times$ PCR buffer, 1.5 mM magnesium chloride and 25 μM each dNTP. The primers amplifying a 262-bp fragment were HPLC-purified. The AS forward primer, 5'-GTG CCT GTC CTG GGA GAG **TT**-3', contains the CC $\rightarrow$ TT tandem transition at the 3' OH site (in bold). Together with the AS reverse primer, 5'-CCT CAT TCA GCT CTC GGA ACA-3', a concentration of 0.1 μM for both primers was used. The amplification was first performed at various temperatures between 60° and 66°C to optimize the annealing conditions. The final amplification program was as follows: 96°C for 2 min, 30 $\times$ (64°C for 1 min, 72°C for 1 min and 94°C for 30 s) and 72°C for 20 min. The entire contents of the AS-PCRs were loaded onto a 1.5% agarose TBE (0.1 M Tris, 0.1 M Borate, 2.5 mM EDTA, pH 7.9) gel and capillary-blotted after electrophoretic separation on a nylon membrane. Following UV crosslinking, the blot was hybridized with a digoxigenin (Dig)-labeled oligonucleotide (0.2 pmol) at 40°C overnight. After incubation with a conjugate

Figure 1. Sensitivity of the AS-PCR. DNA of the mutant cell line HaCaT was serially diluted with WT keratinocyte DNA and then assayed with AS-PCR. The signals shown were detected with the nonradioactive Lumi-Imager system after Southern blotting and hybridization with a Dig-labeled oligonucleotide. The exposure time was 10 min. Signal areas of the same size in each lane were compared for their signal intensity after background correction. The diagram shows the average-fold intensity over the negative control reaction (= 1) of the different dilutions of mutant DNA. Lane a: 0.001% mutant alleles, lane b: 0.01% mutant alleles, lane c: 0.1% mutant alleles and lane d: 1% mutant alleles. The signals in lanes b, c and d are significantly higher than the control and are significantly different from each other ($P < 0.05$ in an unpaired, one-tailed Student's *t*-test).

Figure 2. Detection of a UV-B-generated point mutation in the tumor-suppressor gene *p53* in primary keratinocytes by AS-PCR. Irradiated keratinocytes (single dose 7.3 mJ/cm²) and the unirradiated control keratinocytes were cultured for three weeks following irradiation. Lane a: unirradiated control keratinocytes, lane b: UV-B-irradiated keratinocytes.

of an anti-Dig Fab fragment and alkaline phosphatase, the PCR products were visualized with the CPD-StarTM Chemiluminescent Reagent (Boehringer Mannheim) according to the manufacturers instructions. The light emission at a wavelength of $\lambda = 466$ nm was detected with the Lumi-ImagerTM system (Boehringer Mannheim) at various exposure times between 30 s and 10 min. No visible PCR product was detected from the unirradiated WT keratinocyte DNA using annealing temperatures between 60° and 66°C. At a temperature of 64°C, the PCR product signal from mutant template (HaCaT DNA) had the highest intensity (data not shown), so that this temperature was chosen as the annealing temperature for the following experiments. Next, we determined the detection limit of the AS-PCR step. Therefore, the WT keratinocyte DNA was serially diluted with mutant genomic HaCaT DNA in a range of 1%–0.001%, corresponding to

1000–1 copies of mutant genomic DNA within 100 000 copies of WT genomic DNA. Correlation between the number of cells and the content of both HaCaT and keratinocyte DNA was achieved by using DNA preparations with very similar yields (ca. 4.5 μ g/10⁵ cells lysed). The negative control reaction contained only WT keratinocyte DNA. Although the HaCaT cell line is known to be aneuploid, the relation between the examined alleles is the same. HaCaT is tetraploid in chromosome 17, on which the *p53* gene is located. However, the described mutation is heterocygote (6). Thus, a mutant HaCaT cell provides as many mutant alleles as the normal keratinocyte WT ones. Then, the AS-PCR assay was performed as described above. Figure 1 shows the signals from serial dilutions of mutant DNA in WT DNA. As a result, the signal intensity of the serial dilutions increases with the content of mutant DNA. The detection limit is reached with the dilution step containing 0.01% mutant alleles. The following dilution (0.001% mutant DNA) did not show signal intensities significantly higher than the WT control in all assays (data not shown). The signal intensity of the other dilutions increases, approximately doubling, with a tenfold higher content of mutant alleles. The increase was determined to be 5.0-, 11.2- and 21.5-fold higher than the control signal using the contents of mutant DNA of 0.01%, 0.1% and 1%, respectively. Next, we determined if the point mutation could be generated by UV-B irradiation in normal human keratinocytes and detected with the AS-PCR method. Figure 2 shows that in irradiated keratinocytes, a DNA fragment of the correct size could be detected, whereas it could not be detected for the unirradiated control cells. Compared with the data of the same blot shown in Figure 1, the order of magnitude of the generated point mutation is between 0.1% and 1%.

This AS-PCR method was successfully applied to detect small amounts of mutant DNA in a high background of WT DNA. Furthermore, we show that the nonradioactive imaging system is suitable to compare the signal intensity of AS-PCR products in a semiquantitative way. Using the stable mutation of the HaCaT cell line as a defined posi-

tive control in serial dilutions, it is possible to determine the order of magnitude of the frequency of the same UV-B-generated point mutation in keratinocytes.

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