Modified ASRA facilitates the characterization of activating point mutations in tumors, in which cancer cells constitute only a minor part of the investigated tissue.

T Papp, M Jafari, U Diener, et al.

Genome Res. 1993 3: 125-126
Access the most recent version at doi:10.1101/gr.3.2.125

Modified ASRA Facilitates the Characterization of Activating Point Mutations in Tumors, in Which Cancer Cells Constitute Only a Minor Part of the Investigated Tissue

Thilo Papp, Mehrdad Jafari, Ulrike Diener, Nathaniel Dudley, and Dietmar Schiffmann[1]

Institute of Toxicology, University of Würzburg, W-8700 Würzburg, Germany; [1]Institute of Animal Physiology, Cytopathophysiology Unit, 18051 Rostock, Germany

Allele-specific restriction analysis (ASRA) represents a rapid screening method for the detection of activating point mutations in oncogenes without the necessity of using radioactivity. Tumor DNA is amplified by PCR using mismatch primers that induce an artificial restriction site at the target codon in the normal, but not in the mutated allele.[1] The mutation is visualized as a persistent undigested fragment after restriction enzyme cleavage and agarose gel electrophoresis. Finally, the accurate sequence of the mutated allele can be determined by direct sequencing of the PCR product, and the mutation is demonstrated as an additional band in the sequencing ladder.

In practice, however, tissue samples frequently constitute a mixture of malignant and normal cells in various ratios. Surgically removed specimens often contain only a minor fraction of cancer cells, whereas most of the tissue is composed of normal cells from surrounding tissue or infiltrating lymphocytes. Such tumor samples often yield almost invisible persistent fragments in ASRA, which cannot be resolved by subsequent sequencing. This occurs because the mutation is blotted out by unspecific background signals of the normal allele, due to the fact that the quantity of the mutant allele is negligible.

In this slightly modified ASRA technique, the sequence of a minor constituent can be determined rapidly and accurately. After digestion, the faint residual fragment is excised, electroeluted, and reamplified under identical reaction conditions, which means a selection of the mutant allele against the normal one. In the case of a mutation, a second digestion generates a strong residual fragment and a definite signal in the sequence ladder of the corresponding PCR product. Because the modified ASRA technique has proven to be a method of high reproducibility, repetitions of the experiment have confirmed the discovered mutation, thereby ruling out possible polymerase errors. As an example, we demonstrate this technique by using a primer pair that is useful for the detection of activating point mutations in the first and second nucleotides of codon 61 of the human N-*ras* oncogene.

MATERIALS AND METHODS

General PCR Conditions

All PCR reactions were performed in a 100-μl volume according to standardized basic conditions[2] containing 50 mM KCl, 10 mM Tris-HCl (pH 8.4 at room temperature), 1.5 mM $MgCl_2$, 100 μg/ml of gelatin, 200 μM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP), and 2.5 units of *Taq* polymerase using varying amounts of template and primers. The samples were denatured at 95°C for 3 min, cycled 30× at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and finally incubated at 72°C for 5 min in a DNA Thermal Cycler (Perkin-Elmer Cetus Instruments). Ten percent of the PCR product, along with the 123-bp ladder as a marker, was resolved on a 3.5% NuSieve and 1% agarose gel and visualized by ethidium bromide fluorescence.

Centricon 30 Purification

Unincorporated dNTPs, PCR primers, and salts were removed by washing three times with 1.5 ml of deionized, distilled water in Centricon 30 concentrators (Amicon, Beverly, MA) at 5000*g* in a 34° angle rotor. Purified PCR products (40 μl) were stored at −20°C.

Electroelution

Indigestible residual fragments were carefully excised and electroeluted in a HSB Elutor (Biometra) with 1× TAE buffer for 1 hr at 100 V and trapped in 70 μl of 3 M sodium acetate (pH 6) with subsequent Centricon 30 purification.

Screening Strategy

Preamplification

To increase the available amount of examination material, 0.1 μg of the genomic tumor DNA was amplified with 300 ng of N5′61 (sense oligonucleotide) and N3′61 (antisense oligonucleotide), respectively. Ten percent of the preamplification product was analyzed on an agarose gel, and 1–5 μl was diluted with 1 ml of distilled water.

ASRA

A 10-μl aliquot of the diluted preamplification product was amplified with 300 ng of N61mA (mismatch primer) and N3′61, respectively. Ten percent of the amplification product was gel analyzed, before subsequent Centricon 30 purification. An appropriate aliquot was digested

3:125–126©1993 by Cold Spring Harbor Laboratory Press ISSN 1054-9803/93 $5.00

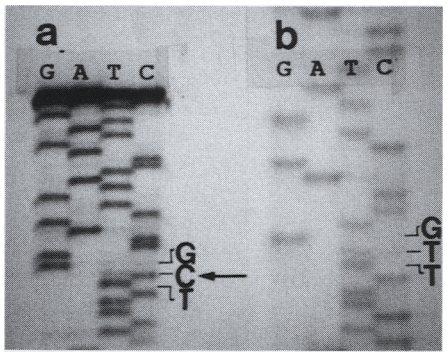

FIGURE 1 Direct sequencing of asymmetric amplified PCR products of the same tumor sample with Sequenase 2.0, ^{35}S, and antisense oligonucleotide SN61 as sequencing primers (see Fig. 2) (*a*) The electroeluted modified ASRA product was amplified asymmetrically with N61mA and sequenced with SN61. Because of the selection of the mutant allele against the normal one, the TTG → TCG transition at the second position of codon 61, converting the amino acid Gln to Arg, is clearly visible. (*b*) The electroeluted preamplification product was amplified asymmetrically with N5′61 and sequenced with SN61. Because the quantity of the mutant allele was too small, only the sequence of the normal allele was visible.

with 10 units of *Msc*I and resolved by gel electrophoresis.

Modified ASRA

The faint residual fragment was carefully excised and electroeluted. Ten percent of the eluate was reamplified under identical reaction conditions. Ten percent of the amplification product was gel analyzed before subsequent Centricon 30 purification. An appropriate aliquot was again digested with 10 units of *Msc*I and resolved by gel electrophoresis. In the case of a mutation, the 85-bp fragment will become indigestible.

Asymmetric PCR and Sequencing

A single-stranded sequencing template was generated by performing an asymmetric PCR with 10% of the purified reamplification product and 900 ng of N61mA, and purified by electroelution. Fifty percent of the eluted single strand was sequenced by means of 30 ng of SN61 (antisense oligonucleotide), with the Sequenase 2.0 kit (U.S. Biochemical) and [α-^{35}S]dATP in accordance with the recommendations of the supplier (for details, see Figs. 1 and 2).

ACKNOWLEDGMENTS

N.D.'s stay in Germany was supported by grants from the Howard Hughes Medical Institute (administered by Hampshire College, Amherst, MA) and The Foremost McKesson Corporation, San Francisco, CA.

REFERENCES

1. Todd, A.V., C.M. Oreland, T.J. Radloff, H. Kronenberg, and H.J. Oland. 1991. Analysis of N-ras gene mutations in acute myeloid leukemia by allele specific restriction analysis. Am. J. Hematol. **38:** 207–213.
2. Erlich, H.A. 1989. PCR technologies: Principles and applications for DNA amplification. Stockton Press, New York.

Received May 24, 1993; accepted in revised form August 3, 1993.

```
        sense oligos                antisense oligos

        amplification primers for exon 2 of the N-ras gene:
N5′61   GTTAT′AGATG′GTGAA′ACCTG     N3′61   ATACA′CAGAG′GAAGC′CTTCG
        amplification product: 112bp

        mismatch primer codon 61:
N61mA   GACAT′ACTGG′ATACA′GCTGG′C   N3′61   ATACA′CAGAG′GAAGC′CTTCG
        amplification product:  85bp
        restriction enzyme:           (MscI TGG!CCA; codon 61)
        digested normal allele: 65bp and 20bp
        undigested mutant allele: 85bp

        sequencing primer:          SN61    AGCCT′TCGCC′TGTCC′TC

           Exon 1 >< Exon 2              +-----------N5′61-
        CCACCATAGAGGATTCTTACAGAAAACAAGTGGTTATAGATGGTGAAACC
        ---------+---------+---------+---------+---------+
        GGTGGTATCTCCTAAGAATGTCTTTTGTTCACCAATATCTACCACTTTGG

        ->       +-------------N61mA-C
        TGTTTGTTGGACATACTGGATACAGCTGGACAAGAAGAGTACAGTGCCAT
        ---------+---------+---------+---------+---------+
        ACAAACAACCTGTATGACCTATGTCGACCTGTTCTTCTCATGTCACGGTA
                                    #61
                                    Gln

        GAGAGACCAATACATGAGGACAGGCGAAGGCTTCCTCTGTGTATTTGCCA
        ---------+---------+---------+---------+---------+
        CTCTCTGGTTATGTACTCCTGTCCGCTTCCGAAGGAGACACATAAACGGT
                    <-SN61----------+
                                    <-N3′61------------+
```

FIGURE 2 Human N-*ras* gene exon 2 with amplification primers (N5′61 and N3′61), mismatch primer (N61mA) with corresponding artificially created *Msc*I restriction site (TGG!CCA) and sequencing primer (SN61). TTG → TCG transition in the second position of codon 61 eliminates the artificial restriction site and leads to an undigested fragment, which corresponds to the mutated allele.