DETECTION OF ING1 GENE MUTATION BY PCR-SSCP TECHNIQUES AND DNA SEQUENCING.

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Abstract

Tumor specific mutation of the candidate tumor suppressor gene ING1 was demonstrated using PCR-SSCP and DNA sequencing analysis. The mutant sample showed an amino acid change from asparagine (AAC) to serine (AGC) at codon 216 in the ING1 gene from the DNA of head and neck cancer cells. The result proved that PCR (polymerase chain reaction) – SSCP (single-strand conformation polymorphism) analysis can effectively detect both wild and mutant types of the gene from tissue samples. Moreover, the nucleotide change can be determined by sequencing the mutant product. The purpose of this report is to illustrate the importance of SSCP and DNA sequencing in identifying nucleotide substitutions in human head and neck carcinomas.
Introduction

The methods of mutation detection can be broadly divided into two categories: testing for known mutations, and screening segments of DNA for possible but unknown mutations.\(^1,2,3,4,5,6\)

Uncontrolled cellular proliferation is a hallmark of cancer. However, cancer is a multistep process where singular gene alterations are necessary but not sufficient for carcinogenesis. Cancer is more likely with additional dysfunction, i.e. inappropriate activation or silencing of the tumor suppressing genes (anti-oncogenes) which control the critical events of the cell cycle, including the process of DNA repair and apoptosis. Abnormalities in genes that regulate the cell cycle can be found in many types of cancer including those of the head and neck region.\(^7\)

ING1 gene has recently been cloned as a novel candidate of tumor suppressor gene.\(^8\) It is reported that overexpression of ING1 can arrest the cells in the G0/G1 phase of the cell cycle, while chronic expression of ING1 antisense constructs promote the cell transformation.\(^9\) In the head and neck squamous cell carcinomas, three missense mutations and three silent changes of ING1 gene have been previously detected in 23 tumors with allelic loss at chromosome 13q33-34 region (10). The purpose of this report is to illustrate the importance of SSCP (single-strand conformational polymorphism) and DNA sequencing in identifying nucleotide substitutions of the ING1 gene in human head and neck squamous cell carcinomas.

The principle of PCR-SSCP and DNA sequencing analyses

PCR-SSCP analyses: A denatured single-stranded DNA makes a stable conformation depending on the DNA sequences, while the conformational structure can be changed by polymorphism or a mutation in the DNA sequences, such as a point mutation, a deletion or an insertion (Fig.1). This is called single-strand conformation polymorphism (SSCP). The mutation in the DNA amplified by PCR is detected due to different mobility of the single-stranded DNA according to the structural change in polyacrylamide gel.

DNA sequencing: For DNA synthesis from a primer against the template DNA, DNA polymerase and four deoxyribonucleotide triphosphates (dATP, dGTP, dCTP and dTTP) are required (Fig.2). Addition of nucleotide analogues, dideoxyribonucleotide triphosphates (ddATP, ddGTP, ddCTP and ddTTP), into the reaction mixture, can gradually terminate the DNA synthesis by incorporating the nucleotide analogues, leading to accumulation of various lengths of DNA fragments with a dideoxyribonucleotide at 3’ end. On ABI PRISM 377 DNA Sequencing System, the four dideoxyribonucleotides are labeled with four fluorescent dyes that emit light at different wavelengths when excited by laser light. All four colors in the sequencing reaction are detected and distinguished in a single lane on the gel, and the DNA sequences are analyzed automatically.

Material and Method

1. PCR amplification of human ING1 gene from normal and tumor tissues.

Tumor suppressor gene ING1 was amplified by PCR with normal or tumor cDNA from human head and neck tissues. PCR mixture contained 100 ng of DNA, 1X PCR buffer, 200 μM of each dNTP, 20 pmole of ING1 S6 and AS3, and 1.25 units of Taq DNA polymerase (Takara, Tokyo, Japan) in 50 μl volume. Initial denaturation
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at 94°C for 3 min was followed by 35 cycles of a denaturation step at 94°C for 30 sec, an annealing step at 64°C for 1 min and an extension step at 72°C for 1 min. A final extension step at 72°C for 7 min was added.

2. SSCP analysis

One µl PCR product was mixed with 8 µl loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) and 1 µl SYBRGreen, heat denatured, chilled on ice and applied on an 8% polyacrylamide gel. The DNAs were electrophoresed in 0.5 x TBE buffer (0.045M Tris-borate, 1 mM EDTA, pH8.0) at 100V for 16 hrs at 15°C.

3. Detection of DNA bands by SYBRGreen

The glass plates were washed with water and rinsed with isopropyl alcohol. DNA stained with SYBRGreen was detected on fluorescent imaging analyzer (Fujifilm, FLA-3000G). An aberrant or shifted band from tumor DNA compared with the normal one was taken to indicate a possible mutation which needs confirmation by DNA sequencing analysis.

4. Purification of the PCR products

PCR products were purified by GeneClean (BIO101, Vista, USA) to remove primers and dNTPs. Forty µl of the PCR products was mixed with 150 µl of 6M NaI, 15 µl of TBE buffer and 10 µl of glass beads for 5 min. After centrifuging at 6,000 rpm for 2 min at room temperature, the supernatant was discarded carefully. The beads were rinsed three times with 300 µl of washing solution, DNA was eluted in 10 µl of elution buffer, and transferred to a new sample tube following centrifuging at 12,000 rpm for 5 min at room temperature.

5. DNA sequencing

Sequencing reaction mixture contained 2µl of purified DNA, 4 µl of Big Dye premix (Perkin-Elmer Applied Biosystems), 2 µl of 5x dilution buffer, 4 µl of ING1 primer (0.8 pmole/µl) and 8 µl of deionized water in 20 µl volume. The sequencing reaction was performed by PCR with 25 cycles of a denaturation step at 96°C for 10 sec, and extension step at 62°C for 4 min. Upon this reaction, fluorescent-labeled deoxyribonucleotides were incorporated into the DNA fragments. After the sequencing reaction, PCR product was mixed with 2 µl of 3M sodium acetate and 50 µl of 95% ethanol, kept at room temperature for 15 min, and centrifuged at 12,000 rpm at room temperature for 20 min. After discarding the supernatant, the DNA precipitate was rinsed with 250 µl of 75% of ethanol, centrifuged at 12,000 rpm at room temperature for 5 min. Again, the supernatant was discarded carefully, and the DNA was dried up. The DNA was dissolved in 4 µl of loading buffer (89% formamide- 4.2mM EDTA-8.3 mg/ml blue dextran), heated at 95°C for 2 min. One µl of the DNA was applied on 6 M urea-5% acrylamide gel, and electrophoresed in 1 x TBE buffer at 1,200 V, 10 hrs on ABI PRISM377 sequencer (Perkin-Elmer Applied Biosystems). On the next day, the sequencing data was analyzed automatically.

Results and Discussion

The tumor suppressor gene ING1 from normal cells and tumor cells of a human head and neck squamous cell carcinoma was searched for mutations by the basic
Fig. 1 Single-Strand Conformation Polymorphism Method

Fig. 2 Sequencing method

Fig. 3 SSCP analysis and sequencing of Normal and Mutated human ING1 Gene

Fig. 4 SSCP analysis of Normal and Mutated human ING1 Gene
methods for gene analysis. Here, detection and sequencing of mutations of the ING1 gene were demonstrated by PCR-SSCP and DNA sequencing analysis. Briefly, human ING1 gene was amplified by PCR. The PCR products were analyzed by PCR-SSCP and DNA sequencing. The PCR-SSCP analyses showed the shift bands of possible mutations in tumor DNA (Fig. 3). Furthermore, DNA sequencing detected the mutated nucleotide (Fig. 4). The mutation showed an amino acid change from asparagine (AAC) to serine (AGC) at codon 216 in the ING1 gene from tumor DNA.

It has been observed previously that up to about 70% of tumors of head and neck squamous cell carcinomas show loss of heterozygosity (LOH) at the chromosomal site 13q33-34, where the ING1 gene is located. Of all LOH cases, missense point mutation frequency of 13% has been reported. The mutation sites include the codon 216 in the exon 2 area of the ING1 gene, which encodes nuclear proteins with a PHD zinc finger in their COOH-terminal half (10). This mutation may particularly affect the conformation of the zinc finger region, preventing the normal function of the ING1 protein.

Additional analyses such as gene expression experiments are required to investigate in more detail how the observed mutation may contribute to the onset and progression of tumors. However, other alterations of the ING1 gene than point mutations are more frequent and hence also important in carcinogenesis.

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