Determination of Tyrosinase mRNA in Melanoma by Reverse Transcription-PCR and Optical Mirror Resonance Biosensor

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Abstracts Tyrosinase transcript in the blood is known as the marker of malignant melanoma and it has been often determined by using reverse transcription-polymerase chain reaction (RT-PCR). However, after the PCR process, the quantification of amplified cDNA by the gel electrophoresis is not reliable and time-consuming. For this reason, we tried to quantify the PCR product using a cuvette-type biosensor, where the oligonucleotide probe was immobilized on the cuvette surface and the single strand cDNA, the denatured PCR product, was then hybridized onto the immobilized probe to give a response signal. The response was immediate and takes 15 min to obtain a stable signal. The biosensor was much more sensitive comparing to the gel electrophoresis method. The quantification of PCR product using a cuvette-type biosensor was feasible and rapid.

Keywords: tyrosinase, determination, melanoma, RT-PCR, optical mirror resonance biosensor, immobilized oligonucleotide probe, transcript

INTRODUCTION

A quantitative analysis of tyrosinase transcripts in the blood of melanoma patients using RT-PCR was introduced as an assay method for melanoma by Smith *et al.* in 1991 [1]. The tyrosinase gene has been used for these studies because the enzyme is involved in the melanogenesis by melanocytes localized in the skin [2-5]. At the time of diagnosis, 10–14% of patients with primary malignant melanoma were found to have detectable number of melanoma cells in blood with this technique [6]. Farmen *et al.* [7], who performed nested primer analysis on duplicates of each of two separated RNA preparations, did also obtain a definitive results in 98.4% of 123 melanoma patients examined. However, their attempts to define the final results as either positive or negative illustrate the need for quantitative analysis of tumor-specific mRNA. Generally, the result of PCR is usually visualized by the gel electrophoresis, of which the sensitivity is not satisfactory.

Biosensors are now widely used to study biomolecular interactions between proteins and peptides, DNA, lipids, carbohydrates, drugs and even cells and are suitable for a range of research application including DNA hybridization. The most widely used instrumental biosensors to date have been the BIAcore range and IAsys systems [8]. The IAsys sensors are cuvette-based systems, which use a waveguide technique called a prism coupler or resonant mirror for detection. The IAsys sensor surface is the bottom of an independent two-well format micro-cuvette, which has a choice of derivatized surfaces for ligand immobilization. If one molecule in free solution binds to or dissociates from the other molecule immobilized on the sensor surface, the binding and dissociation events can be seen as shifts in resonance angle (Fig. 1).

In this study, we have carried out rapid, unlabeled single stranded DNA-DNA hybridization analysis on IAsys using a biotinylated oligonucleotide attached to the sensor surface via streptavidin [9]. The specific hybridization of unlabeled PCR product from cultured melanoma cells to the captured oligonucleotide probe was measured at 25°C and detected within seconds of adding the sample. The sensitivity of sensor was studied by changing PCR product size and concentration.

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MATERIALS AND METHODS

Materials

IAsys carboxymethyl(CM) dextran coated cuvettes, *N*-ethyl-*N'*-dimethylaminopropyl-carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) coupling chemistry kit was obtained from Affinity Sensors Co. (Cambridge, UK). Streptavidin (affinity purified) was purchased from the Sigma Chemical Co. (MO, USA). The primer sequences for the amplification of tyrosinase mRNA are as follows,

(a) product size: 65 bp
forward: CCTCCTGGCAGATCATTTGT (position; 911-930)
reverse: ATCGCATAAAACCTGATGGC (position; 951-970)

(b) product size: 250 bp
forward: CCTCCTGGCAGATCATTTGT (position; 911-930)
reverse: GGCAAATCCTTCCAGTGTGT (position; 1127-1146)
Beta-actin was used as a non-specific PCR product, of which sequence is,
forward: GTGGGCCGCCCTAGGCACCAG
reverse: CACTTTGATGTCACGCACGATTTC
We also used a competitor(see below), of which sequence is,
CTGATCTGCTACAAATGATCTGCCAGGAGG (position; 943-972)

Melanoma Cell Culture

B16/F10 mouse melanoma cells were cultured in Dulbecco's modified essential medium (DMEM) with 5% bovine calf serum and 1% penicillin/streptomycin. Cells were grown to confluence, harvested with 1x trypsin-EDTA solution.

RT-PCR

Total RNA was isolated using RNAgents® Total RNA Isolation System (Promega, CA, USA). The quality of RNA was tested by the A_{260}/A_{280} ratio and 1.5% agarose gel electrophoresis. Complementary DNA (cDNA) synthesis was performed with M-MLV reverse transcriptase using approximately 2 μg of total RNA and 100 pmol of oligo-dT_{17} primers in 25 μL reaction mixture. RNA was incubated at 37°C for 60 min, and then heated at 95°C, 5 min. PCR was carried out as described previously [1] with some modifications. Two microgram of cDNA synthesized was used for PCR amplification in 50 μL of reaction mixture containing: 5x PCR buffer (50 mM KCl, 20 mM Tris, pH 8.4), 1.5 mM $MgCl_2$, 10 pmol of each primer, 10 mM dNTP and 0.2 U of recombinant *Taq* polymerase. Each sample was heated at 95°C for 5 min. For the detection of tyrosinase mRNA, 30 cycles of PCR were then carried out (95°C for 50 sec, 56°C for 30 sec, 72°C for 30 sec). PCR products were analyzed by electrophoresis on 1.5% agarose gels, followed by ethidium bromide staining.

Preparation and Immobilization of 5'-Biotin-labelled Probe

Streptavidin was immobilized onto a carboxymethyl dextran cuvette using a mixture of EDC and NHS [9]. Briefly, the cuvette was washed with 50 μL of phosphate-buffered saline with 0.05% Tween 20 (PBST) and equilibrated for 20 min with 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (pH 7.4, 3.4 mM EDTA, 0.15 mM NaCl and 0.005% Tween 20). The surface was activated using two injection of 50 μL of a mixture of 0.05 M NHS and 0.2 M EDC for 10 min. Streptavidin was then injected (50 μL of 200 μg/mL solution in 10 mM sodium acetate, pH 4.5) for 60 min. Residual active sites were blocked with 1 M ethanolamine, pH 8.5. The biotinylated oligonucleotide probe, of which sequences are the same as forward primer (biotin-911~930; 20 bp), was purchased from Takara (Seoul, Korea). Twenty μL of biotinylated probe (400 μg/mL solution) was added to 45 μL PBST in the cuvette and allowed binding to occur for about 300 sec.

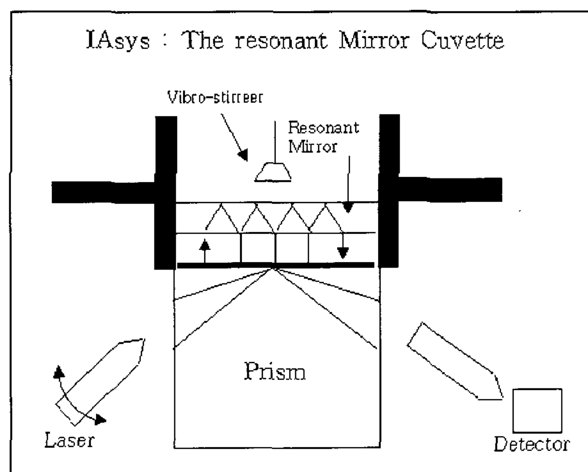

Fig. 1. The principle of optical mirror resonance type biosensor with cuvette; binding and dissociation events are seen as shifts in resonance angle as one molecule in free solution binds to the other molecule immobilized on the sensor surface.

Hybridization Test Using Biosensor

PCR generated double strand cDNAs were denatured into single strand cDNA by rising temperature up to 95°C and then by quenching them into 4°C. For hybridization test, IAsys plus system (Affinity Sensors Co., UK), a cuvette-based optical biosensor, was used. PBST buffer was placed in the cuvette and replaced with 5 μL of PCR single strands (20 μg/mL in PBST) in hybridization buffer (5x saline sodium citrate (SSC), 5x Denhardt's solution, 5 mM sodium phosphate, 0.1% v/v Tween 20) and incubated for 15 min. The binding response was monitored, and after a PBST buffer wash, the shift in resonance position was measured. To repeat the hybridization tests, bound cDNA was dissociated from the probe using 0.25 M NaOH solution.

RESULTS AND DISCUSSION

Cuvette Activation and Immobilization of Streptavidin

Streptavidin was immobilized onto the cuvette sensor surface using NHS/EDC chemistry as described in Materials and Methods and the signal change during the procedure is plotted in Fig. 2. The signal obtained by the immobilization of streptavidin was about 2,000 arc seconds (peak 3). The biotin labeled oligonucleotide probe (20 bp) was also immobilized by streptavidin-

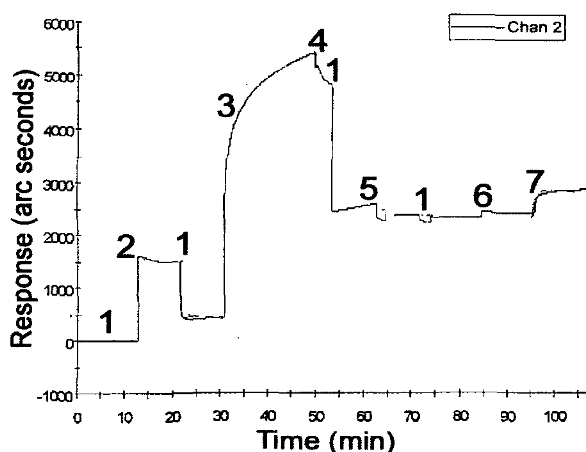

Fig. 2. Cuvette activation and immobilization of streptavidin.
1. Acetate buffer, pH 5.0
2. Activation by EDC/NHS
3. Addition of streptavidin
4. 1 M ethanolamine
5. 20 mM HCl
6. Washing with PBST
7. Addition of biotin-probe

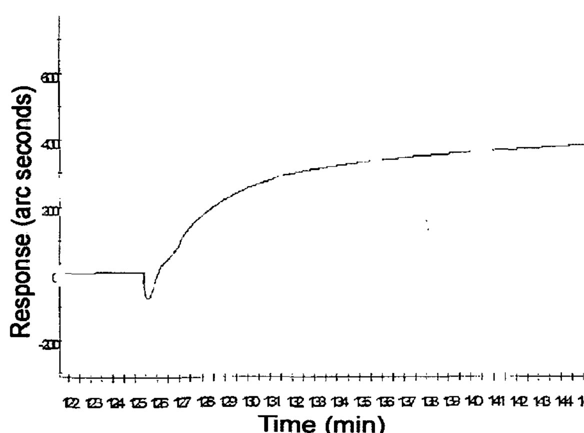

Fig. 3. Hybridization with single strand cDNA of 65 bp.

biotin interaction and the obtained signal was about 200 arc seconds (peak 7). The large difference in amplitude of signals between the streptavidin and biotin-probe may be due to the difference of molecular weight, because the sensor signal is deeply influenced by the molecular size of the molecules hybridized to the probe.

Hybridization with Single Strand cDNA

When the single strand cDNA of PCR product was applied to the cuvette, the response signal was observed as shown in Fig. 3. The response was almost saturated within 15 min. But amplitude of signal was less than 400 arc seconds, because the size of cDNA employed in this hybridization test was 65 bp. To increase the amplitude of signal further, we changed the cDNA size or applied a kind of competitor sequence. The role of competitor used here is as follows. To obtain higher signal amplitude in the hybridization test, we have to enhance

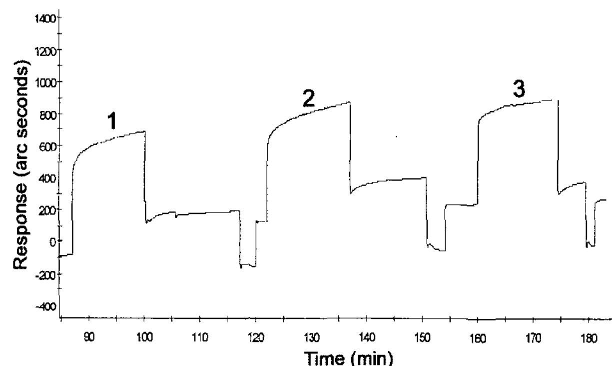

Fig. 4. The effect of cDNA size or competitor on the amplitude of signal: 1: 65 bp, 2: 250 bp, 3: 250 bp with competitor (see text).

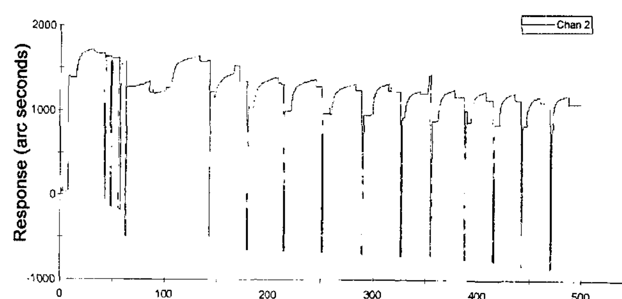

Fig. 5. Repeated hybridization equilibrium. The bound single strand cDNA was dissociated using 0.25 M NaOH solution.

the hybridization between the immobilized probe and the single strand cDNA. However, due to a steric hindrance in the interaction between the probe and the single strand cDNA, there is always a possibility of self-rehybridization between the two single strand cDNA, thus reducing the amount of cDNA hybridized onto the probe. The competitor is an inhibitor against self-rehybridization between the two single strand cDNA, because the competitor sequence can only hybridized onto the amplified forward cDNA, but neither onto the reverse cDNA nor onto the immobilized probe. Fig. 4 shows the hybridization between biotin-probe and the single strand cDNA, 65 bp (peak 1), 250 bp (peak 2) and 250 bp including competitor sequence (peak 3). Differently from our expectation, there was no significant difference in the signal amplitude between 65 bp and 250 bp, which gave about 400 arc seconds. In addition, the effect of competitor was not so significant because the obtained signal was less than 500 arc seconds. Fig. 5 shows the result of repeating hybridization cycle with dissociating the bound cDNA using 0.25 M NaOH solution. In spite of the sharp drop of signal between the cycles due to the change of pH occurred by the addition of NaOH solution, there was practically no change in the signal amplitude even after 10 cycles are repeated. The PCR product was diluted by 100 folds with PBST and applied to the cuvette or to the gel electrophoresis.

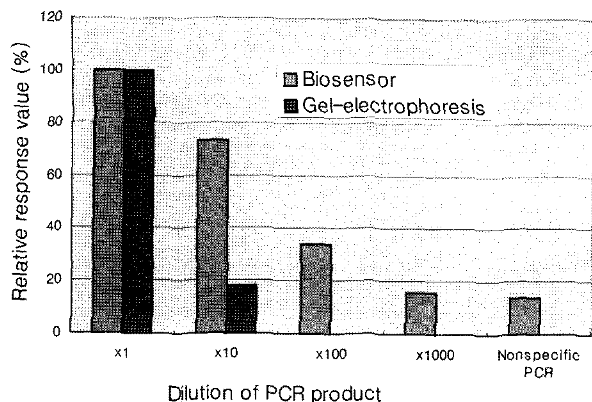

Fig. 6. Relative sensitivity of biosensor or gel electrophoresis to determine the transcripts of tyrosinase in melanoma. The transcript of beta-actin was used as a nonspecific PCR product.

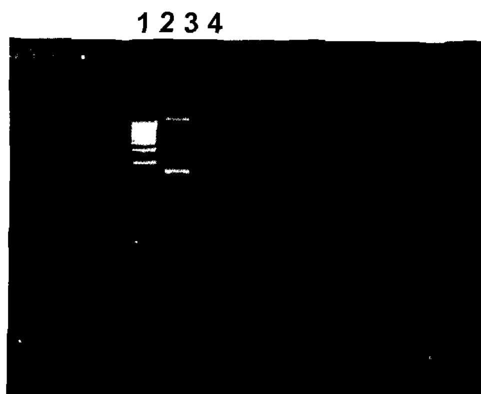

Fig. 7. Gel electrophoresis of PCR product of tyrosinase in melanoma: 1: Molecular weight size marker, 2: 1× dilution, 3: 10× dilution, 4: 100× dilution.

The response signal from biosensor was still significant comparing to that of nonspecific PCR product of beta-actin, while the signal from gel electrophoresis was null. (Fig. 6) The result that biosensor is 100 times more sensitive than gel electrophoresis in the determination of the tyrosinase transcripts in melanoma was also obtained (Fig. 7). These preliminary studies together suggest that biosensor system is more feasible and rapid for the quantification of PCR product, although there are still lots of things to be studied further.

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