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High throughput differential identification of TMPRSS2-ERG fusion genes in prostate cancer patient urine

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ABSTRACT

Identifying genetic diversity is important for studies in cancer as it can provide insights on disease progression and treatment. Although clinical outcome and major symptom of cancer might be same in all patients, the type of overexpressed gene could be different. Even though prostate-specific antigen assay is a good tool widely used for prostate cancer diagnosis, it is not capable of providing information on genetic differences. Therefore, screening method that can differentiate genetic differences is necessary. Here we detected different types of *TMPRSS2-ERG*, prostate cancer specific fusion genes, to verify the genetic diversity between the patients using high throughput screening method, bio-barcode assay. Prostate cancer patients with different types of fusion gene were successfully differentiated directly from untreated patients' urine, while traditional PSA assay could not. This non-invasive assay, when used with PSA assay, can be a strong secondary screening method which can offer new insights on disease progression and clinical outcome.

1. Introduction

Prostate cancer, defined as the most commonly diagnosed cancer in men, is a significant public health issue due to its high incidence rate and cost regarding its diagnosis and treatment [1,2]. Measurement of prostate-specific antigen (PSA) in serum, the representative biomarker for prostate cancer, is a commonly used method for diagnosing and screening prostate cancer [2–4]. However, PSA concentration reaches almost to zero level in urine of patients after hormone therapy and PSA-based screening method alone is insufficient to provide information on genetic diversity. The patient-friendly diagnostic tool using bodily fluid is preferred, and the discovery of additional biomarker that supplements PSA to gain information on genetic diversity is also required [5,6].

Fusion genes have been regarded as both a diagnostic tool that can be used to monitor patients and therapeutic targets that will be subject to eventual treatment [7]. Fusion genes are generated in various diseases including leukemia, sarcoma, and carcinoma through chromosomal rearrangement [8]. The rearrangement of genes causes a genomic instability in cells, which results in an increased risk for the disease [7]. The prostate cancer overexpresses a specific fusion gene, *TMPRSS2-ERG*, which has a role in tumor progression including invasion and metastasis [9]. *TMPRSS2-ERG* fusions are the most predominant isoform, with multiple studies showing that approximately 50% of prostate cancers from PSA screened surgical cohorts are *TMPRSS2–ERG* fusion-positive, and greater than 90% of prostate cancers over-expressing ERG harbor *TMPRSS2–ERG* fusions [10,11].

Current methods for the detection of fusion genes are reverse transcription-polymerase chain reaction (RT-PCR), fluorescent in situ hybridization (FISH) and prior-cDNA microarray [11]. These methods, however, are not cost-effective and inapplicable for multiplexed detection of genes. Although multiplex PCR has been reported and developed recently for diagnosis, multiple targets detection in a single assay is still issue. Because multiplex PCR requires optical primer pairs, and highly optimized PCR condition due to low PCR efficiency [12]. For these reasons, interest in a new

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method with high sensitivity and multiplex capability has been increased recently [13,14]. Among nanomaterial-based bioassays [15–20], a bio-barcode assay, was established that detects proteins and nucleotides with ultrahigh sensitivity [21–23]. Sandwich assay used in this technique allows an exclusive sorting of fusion genes since different sequences on each side are required to contribute to final outcome [22,24–26]. The sandwich assay using nanoparticles and gel electrophoresis can be easily used in any laboratory environment since it does not require large scale expensive equipment.

Here we suggest high throughput electrophoresis-based bio-barcode assay that can function as a model method to determine signature fusion genes in urine of men with prostate cancer. Since the type of fusion genes depends on the phase of prostate cancer progression [11], we focused on three different target fusion genes, which fuse different regions of exon 1 or 2 of *TMPRSS2* to exon 2 or 4 of *ERG*, to achieve diagnosis of a specific phase of prostate cancer. Moreover, determination of fusion gene types in early stage cancers is important for patient therapy and prognosis [27]. Thus, we detected fusion genes in urine samples of patient group with early stage cancer (Gleason score 6 or 7). We adopted a bio-barcode assay to increase the specificity of targeting fusion genes as well as decreasing the non-specific signal from urinary components. Importantly, we demonstrated a successful differential detection of multiple types of fusion genes from urine of patients whose PSA levels are similar. These findings suggest that differential determination of fusion genes from clinical samples can offer new insight into disease progression and clinical outcome.

2. Materials and methods

2.1. Capture DNA-modified magnetic microparticle (MMP) probe preparation

2.8-μm carboxylic acid-functionalized MMP (Dynabeads® M-270 Carboxylic Acid, 14305D, Invitrogen, Carlsbad, California, USA) was vortexed briefly and magnetically separated in a magnetic separation rack (MSR1000, OZ Biosciences, San Diego, California, USA) for 2 min to remove the supernatant. The MMP was then washed twice with 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (M8250, Sigma-Aldrich, St. Louis, Missouri, USA) buffer with pH 5. Amine-terminated DNAs (capture DNAs) (Integrated DNA Technologies, Coralville, Iowa, USA), which enabled the capturing of a target sequence as the binding to a half of the complementary target sequence, were prepared in a MES buffer, mixed with MMP and shaked at room temperature for 30 min.

The capture DNA and the MMP were cross-linked with each other by adding 100 mg/ml *N*-(3-Dimethylaminopropyl)-*N'*-ethyl-carbo-diimide hydrochloride (EDC) (03450, Sigma-Aldrich) at room temperature. After 3 h of shaking and magnetic separation, the supernatant was removed, and the absorbance of the supernatant was then measured using UV–Vis spectrophotometer (S-3100, SCINCO, Seoul, South Korea) at 260-nm wavelength to calculate the amount of the capture DNA conjugated to MMP. The MMP was washed with 50 mM Tris-HCl buffer adjusted to pH 7.4 (15568-025, Invitrogen, Carlsbad, California, USA) by 10 min of shaking, and this washing step was repeated 2 times. Finally, DNA-functionalized MMP was resuspended in phosphate-buffered saline (PBS) with pH 7.4 (10010-023, Invitrogen, Carlsbad, California, USA) at a final concentration of 30 mg/ml.

2.2. Probe DNA-modified gold nanoparticle (AuNP) probe preparation

10 nmole of oxidized thiol-terminated oligonucleotides (probe DNA) (Integrated DNA Technologies) were reduced by 100 mM 1,4-dithiothreitol (DTT) (H7033, Sigma-Aldrich), which was prepared in a phosphate buffer with pH 8 to remove the protecting group from the oligonucleotides. After 1 h incubation at room temperature, deprotected probe DNA was applied to illustra™ NAP™-5 Columns (GE17-0853-01, Sigma-Aldrich, St. Louis, Missouri, USA) for purification of the probe DNA from excess DTT. The probe DNA was then mixed with 50-nm gold nanoparticle (AuNP) solution (EM.GC50, BBI Solutions, Cardiff, UK), and the mixture was pre-incubated at 37 °C for 1 h. Phosphate buffer with pH 7 and 10% sodium dodecylsulfate solution (V6551, Promega, Fitchburg, Wisconsin, USA) was added for pH adjustment, followed by the addition of six aliquots of 2 M sodium chloride solution (S7653, Sigma-Aldrich, St. Louis, Missouri, USA) to a final concentration of 0.3 M. The resultant solution was then incubated at 37 °C overnight. Probe DNA-functionalized AuNP was centrifuged at 8500 rpm for 15 min, and concentrated in deionized water which is of 1/10 volume of initially used AuNP solution.

2.3. Quantification of the amount of probe DNA conjugated to AuNP

To quantify oligonucleotides fluorescently, probe DNA modified with Alexa Fluor-488 fluorophore (Integrated DNA Technologies) was used. The procedure of preparation of fluorophore-labeled probe DNA-modified AuNP is same as the procedure described above, except for the protection from the light to prevent photobleaching of the fluorophore. The fluorophore-labeled probe DNA-functionalized AuNP was centrifuged at 8500 rpm for 15 min and resuspended in 1 ml of deionized water to remove unbound DNA (repeated twice). After additional centrifugation, the pellet of fluorescence-labeled AuNP probes was treated with 50 μl of 25 mM potassium cyanide (KCN) (60178, Sigma-Aldrich), and the mixture was then incubated at room temperature until the entire dissolution of AuNP. Fluorescence intensity of Alexa Fluor-488 fluorophore-modified probe DNA was measured using Infinite® F200 Pro (Tecan, Zürich, Switzerland) to obtain the standard curve. The samples were serially diluted in 10-fold increments ranging from 10 μM to 1 nM. Finally, fluorescence intensity of the fluorescence-labeled DNA released from AuNP was measured to determine the number of probe DNA cross-linked to AuNP by comparison to the standard curve.

2.4. Bio-barcode assay for detection of fusion genes

Target oligonucleotides (DNA and RNA) were serially diluted in 10-fold increments with the wide range of concentration (10 nM-100 aM). The solution used for samples was prepared by the dilution of a PerfectHyb™ Plus Hybridization Buffer (H7033, Sigma-Aldrich) with 3 times volume of PBS (hybrid buffer). Both 10 μl of magnetic probes and 10 μl of AuNP probes were added to 100 μl of serially diluted samples. The mixtures were vortexed briefly and incubated at 95 °C for 5 min for initial dehybridization of double-stranded nucleotides. The samples were then cooled down on ice for 5 min, followed by shaking for 1 h which allowed the probes and the target nucleic acids to form sandwich complexes. The MMP-target-AuNP complexes were magnetically separated briefly, washed twice with the hybrid buffer, and resuspended in 10 μl of deionized water. The samples were then heated up to 95 °C for 5 min to allow the dehybridization of double-stranded nucleotides, and magnetically separated, and the supernatant, which contained the AuNP probes released from MMP as well as target nucleotides, was transferred to new 0.2 ml tube. To dissolve the AuNP into the ionized form, 2 μl of 100 mM KCN was added to the samples. In addition, 1 μl of 1 μM oligonucleotides (hybrid DNA) complementary to probe DNA and 4 μl of 1 M sodium chloride solution (S7653, Sigma-Aldrich) were added to form double-stranded probe DNA