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Sensitive array-based assay for determination of serological protein kinase A autoantibody levels based on its antigen protein activation

Su-Hyeon Kim [a,1], Se-Hui Jung [a,1], Deok-Hoon Kong [a], Hye-Yoon Jeon [a], Min-Soo Kim [a,b], Eun-Taek Han [c], Won Sun Park [d], Seok-Ho Hong [e], Young-Myeong Kim [a], Kwon-Soo Ha [a,*]

[a] Department of Molecular and Cellular Biochemistry, Kangwon National University School of Medicine, Chuncheon, Kangwon-Do 200-701, South Korea
[b] Department of Anesthesiology, Kangwon National University School of Medicine, Chuncheon, Kangwon-Do 200-701, South Korea
[c] Department of Medical Environmental Biology and Tropical Medicine, Kangwon National University School of Medicine, Chuncheon, Kangwon-Do 200-701, South Korea
[d] Department of Physiology, Kangwon National University School of Medicine, Chuncheon, Kangwon-Do 200-701, South Korea
[e] Department of Internal Medicine, Kangwon National University School of Medicine, Chuncheon, Kangwon-Do 200-701, South Korea

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ABSTRACT

Objectives: We investigated the effect of cPKAα conformational states during protein immobilization on an array platform for cPKA autoantibody assays for sensitive and high-throughput profiling of protein kinase A (PKA) autoantibody levels in human sera.

Design and methods: We prepared activated human cPKAα protein arrays by addition of cofactors including ATP, $MgCl_2$, and Triton X-100 to incubation buffer. Anti-human cPKAα antibody or PKA autoantibody levels in human sera were analyzed using activated human cPKAα protein arrays.

Results: Activation of cPKAα with ATP, Mg^{2+}, and Triton X-100 enhanced the sensitivity of the assay by increasing the signal/noise ratio and lowering the limit of detection. cPKAα activation also enhanced the sensitivity of cPKA autoantibody detection in human sera. We successfully applied this assay to determine cPKA autoantibody levels in human sera from normal individuals (n = 30) and hepatic cancer patients (n = 30).

Conclusions: Our results demonstrate that cPKAα activation enhanced the sensitivity of array-based PKA autoantibody assays, and that this assay is suitable for high-throughput analyses of cPKA autoantibodies in human sera.

1. Introduction

The identification of tumor biomarkers has been a great challenge for past three decades because early diagnosis and detection are critical factors in cancer treatment [1–3]. A variety of tumor biomarkers that are based on protein abundance are currently available, including human epidermal growth factor receptor 2 (HER2) [3] and cancer antigen (CA) 15-3 [4] for breast cancer, carcinoembryonic antigen (CEA) for colorectal cancer [5], CA125 for ovarian cancer [6], CA 19-9 for gastrointestinal cancer [7], α-fetoprotein (AFP) for liver cancer [1], and prostate-specific antigen (PSA) for prostate cancer [8]. Additionally, cancer biomarkers based on enzyme activity, including blood coagulation factor XIII (FXIII) activity for hepatocellular carcinoma (HCC), have also been reported [9,10]. However, because only small amounts of these antigens are released into circulation in the early stage of disease and are rapidly degraded and cleared, these cancer biomarkers have limited clinical applications due to their relatively low diagnostic sensitivity and specificity [3].

Autoantibodies produced by the patient's immune system during exposure to cancer specific antigens have emerged as alternative cancer biomarkers [2,3,8]. The use of autoantibodies as biomarkers can overcome abundance-related limitations because relatively low levels of antigens produce higher levels of autoantibodies in blood [3]. A number of autoantibodies against antigens, including HER2 [11], carbonic anhydrase XII [12], mutated p53, tyrosinase, SOX2, ZIC2, SSX2, MAGE, and NY-ESO [13,14], have been suggested as new potential biomarkers. However, it remains a challenge to overcome their sensitivities. Furthermore, most antigen proteins used for autoantibody assays are recombinant; thus, they lack post-translational modifications [3]. Therefore, additional modifications to antigen proteins that induce conformational states that are similar to their conformations in blood should be helpful for quantifying autoantibodies in serum samples.

Recently, the protein kinase A (PKA) autoantibody emerged as a potential new target for cancer diagnosis [1]. PKA is one of the most important enzymes in several biological processes induced by post-translational protein phosphorylation [1,15–17]. PKA is composed of four subunits, two regulatory and two catalytic subunits, and exists in an inactive state. Activity of the catalytic subunits of PKA (cPKA), which are dissociated from the tetrameric holoenzyme upon binding of four cyclic AMP molecules, is further regulated by allosteric conformational

* Corresponding author.
E-mail address: ksha@kangwon.ac.kr (K.-S. Ha).
[1] S.-H. Kim and S.-H. Jung contributed equally to this work.

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changes induced by ATP, the coordination of metal cofactors bound to ATP, and a heat-stable protein kinase inhibitor [18]. In various types of cancer cells, cPKA is excreted into the extracellular space [19] and its activity is markedly increased in sera from cancer patients, suggesting cPKA is a potential cancer biomarker [19,20]. Moreover, cPKA autoantibody levels are also up-regulated in sera from patients with cancer [1,2]. Thus, the development of a sensitive and high-throughput assay system for evaluating and monitoring serological cPKA autoantibody levels in patients with various diseases, including cancer, is necessary.

In this study, we developed a sensitive and high-throughput PKA autoantibody assay using cPKAα arrays, which were fabricated by immobilizing the antigen protein in an active state on well-type amine arrays, fabricated by mounting Poly(dimethylsiloxane) (PDMS) gaskets onto the amine-modified glass slides. cPKAα activation enhanced the sensitivity of the antigen array-based autoantibody assay in human sera by increasing the signal/noise (S/N) ratio and lowering the limit of detection (LOD) compared to the non-activated control. Thus, we suggest that it is important to activate antigen proteins, including cPKAα, on an array platform for high-throughput profiling of autoantibody levels in human sera.

2. Materials and methods

2.1. Chemical reagents

3-aminopropyltrimethoxysilane and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). cPKA was obtained from Biaffin GmbH & Co. KG (Kassel, Germany). Rabbit anti-human cPKA and Alexa Fluor 546-conjugated anti-rabbit or human IgG were purchased from Abcam (Cambridge, MA) and Invitrogen (Molecular Probes, Eugene, OR), respectively. PDMS solution was obtained from Sewang Hitech (Gimpo, Korea).

2.2. Serum samples

Serum samples collected from normal individuals (n = 30; male = 27 (age, 62.3 ± 13.3) and female = 3 (66.7 ± 18.6)) and patients with hepatic cancer (n = 30; male = 13 (55.7 ± 15.3) and female = 17 (49.6 ± 14.5)), who had AFP levels over 10 ng/ml and were clinically diagnosed as hepatocellular carcinoma, were obtained by the Kangwon National University Hospital Biobank (a member of the National Biobank Korea) and stored at −80 °C. Experiments using human samples were performed with the approval of the local Institute's Ethics Committee for research in human subjects.

2.3. Fabrication of well-type amine arrays using PDMS gaskets

Well-type amine arrays were fabricated as previously reported [21–23]. Briefly, glass slides (75 × 25 mm) were cleaned with a H_2O_2/NH_4OH/H_2O solution (1:1:5, v/v), immersed in 1.5% 3-aminopropyltrimethoxiysilane (v/v) for 2 h, and baked at 110 °C. PDMS prepolymer solution was prepared by mixing 5 g PDMS base and 0.5 g curing agent until it became cloudy with bubbles. Degassing was performed for 30 min. The mixture was poured into a chrome-coated copper mold with arrayed poles (1.5 mm diameter and 0.3 mm height; Amogreen Tech, Kimpo, Korea). The mold was incubated at 84 °C for 90 min, and PDMS gaskets containing arrayed holes of 1.5 mm in diameter were detached. Well-type amine arrays were fabricated by mounting PDMS gaskets onto the amine-modified glass slides.

2.4. Preparation of activated human cPKAα protein arrays

Activated human cPKAα protein arrays were fabricated as follows: Human cPKAα (50 μg/ml) was prepared in 50 mM Tris–HCl (pH 7.5) containing the indicated combinations of 0.5 mM ATP, 0.5 mM $MgCl_2$, and 0.01% Triton X-100. The mixtures were applied to well-type amine arrays at 37 °C for 60 min. The resulting human cPKAα protein arrays were sequentially washed with phosphate-buffered saline (PBS) containing 0.1% Tween-20 (0.1% PBST) for 10 min and Milli-Q water for 5 min.

2.5. Analyses of anti-human cPKAα antibody or PKA autoantibody levels in human sera using human cPKAα protein arrays

Anti-human cPKAα antibody or PKA autoantibody levels in human sera were analyzed using human cPKAα protein arrays. cPKAα arrays were blocked with 1% BSA in 0.1% PBST at 37 °C for 60 min. The indicated concentrations of the rabbit anti-human cPKAα antibody or 20-fold diluted human sera in PBS containing 0.05% Tween-20 (0.05% PBST) were applied to human cPKAα arrays at 37 °C for 60 min. Arrays were probed at 37 °C for 60 min with 10 μg/ml Alexa Fluor 546-conjugated anti-rabbit IgG or anti-human IgG, respectively, in 0.05% PBST containing 1% BSA. Arrays were washed with 0.1% PBST and Milli-Q water, dried under air, and analyzed using a fluorescence scanner equipped with a 543-nm laser (ScanArray Express GX, Perkin Elmer, Waltham, MA).

2.6. Data analyses

ScanArray Express software (Perkin Elmer) was used to quantify fluorescence intensities and for data extraction. Fluorescence intensities of array spots were expressed as signal to noise ratio (S/N) determined by dividing fluorescence intensities of spots treated with commercial cPKAα antibody or human serum samples by those of blank spots. The limit of detection (LOD) of the PKA antibody assay was calculated using the equation: LOD = blank + S.D. × 3, where S.D. is the standard deviation of the blank sample. To compare two populations, we perform *t* tests using the Origin 6.0 software package (Origin Lab, Northampton, MA). p values < 0.05 were considered statistically significant.

3. Results

3.1. Optimization of the PKA antibody assay using human cPKAα protein arrays

We developed an on-chip PKA autoantibody assay for sensitive and high-throughput analyses of cPKA autoantibody levels in human serum samples by activation of its antigen human cPKAα. To determine the optimal human cPKAα concentration for autoantibody assays, we applied the rabbit anti-human cPKAα antibody to antigen protein arrays with protein concentrations ranging from 1 to 100 μg/ml. As shown in Fig. 1A, binding of the anti-human cPKAα antibody to the protein arrays increased in a cPKAα-dose dependent manner, with saturation at 50 μg/ml, suggesting that this concentration is suitable for fabrication of human cPKAα protein arrays.

Binding of the rabbit anti-human cPKAα antibody to cPKAα arrays was probed with a secondary antibody, Alexa Fluor 546-conjugated anti-rabbit IgG. To determine the optimal concentration of the secondary antibody, various concentrations (0.1–50 μg/ml) of the secondary antibody were applied to the cPKAα arrays that were incubated with 20 μg/ml of the rabbit anti-human cPKAα antibody. Saturation of secondary antibody binding was achieved at 10 μg/ml, with a calculated dissociation constant (Kd) of interaction between the primary and secondary antibodies of 0.62 μg/ml (Fig. 1B). This result suggests that Alexa Fluor 546-conjugated anti-rabbit IgG can be used as a probe to analyze anti-human cPKAα antibody bound to the protein array surface. Moreover, these data demonstrated that 10 μg/ml of the secondary antibody is suitable for PKA autoantibody assays.

We next investigated whether this assay is specific to the human cPKA antibody in mixed samples. Various concentrations of human IgG, ranging from 0.1 to 50 μg/ml, were applied to cPKA protein arrays. The arrays were subsequently probed with Alexa546 Fluor-conjugated