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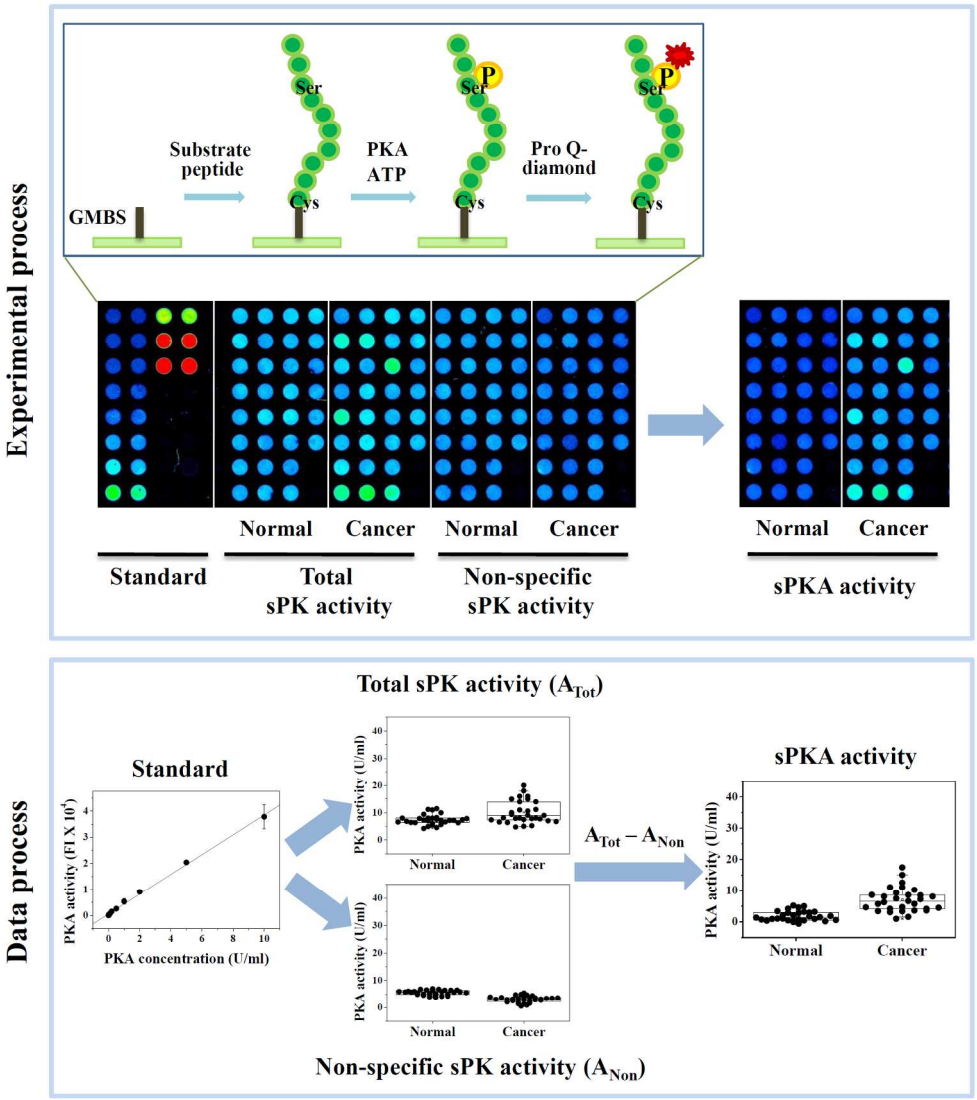
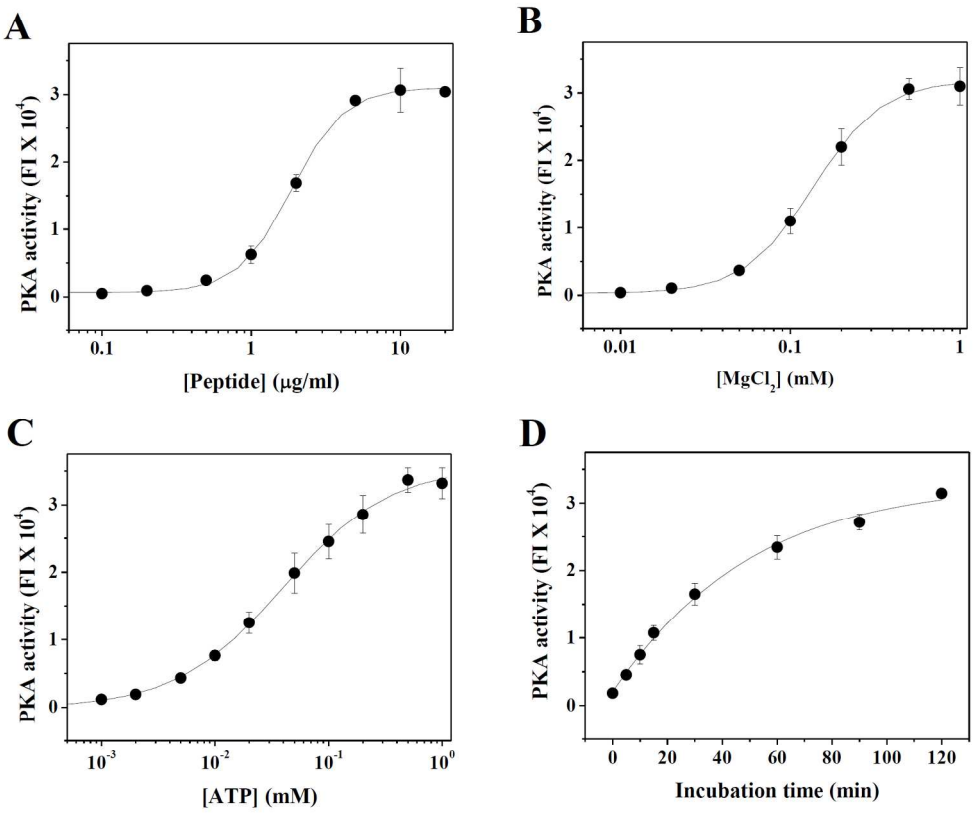
Fig. 1. Schematic diagram of sPKA activity assay in human sera using fluorescence-based peptide arrays.



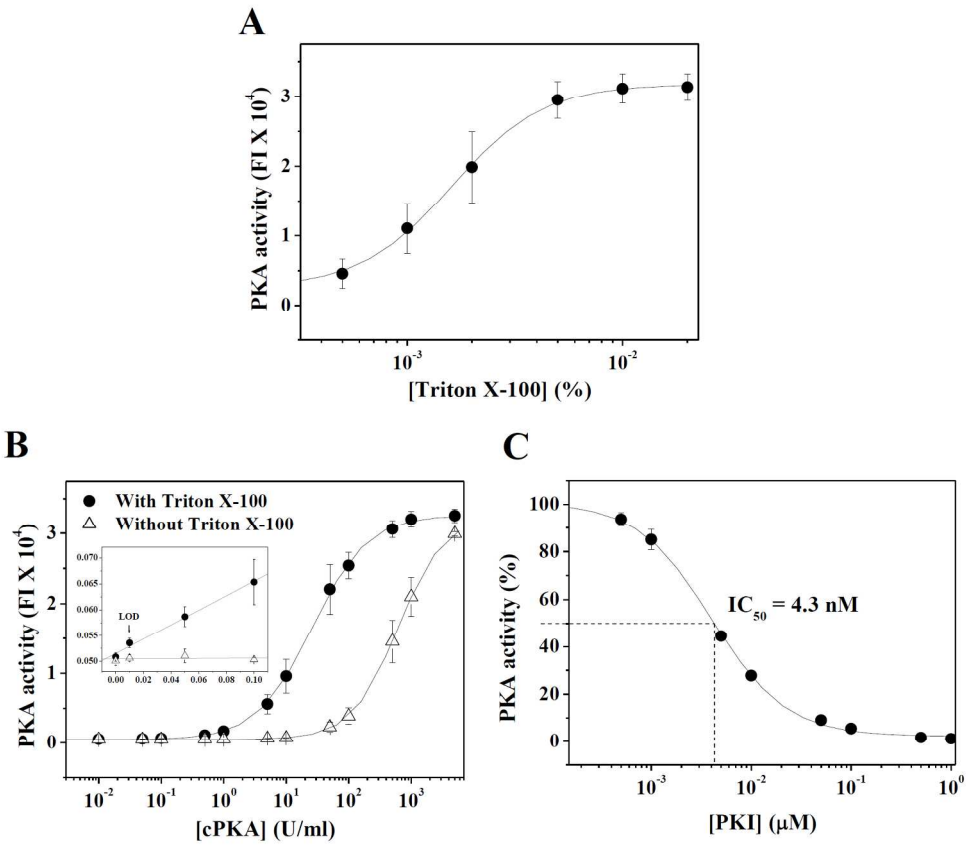
776x948mm (72 x 72 DPI)

Fig. 2. Optimization of on-chip sPKA activity assay.



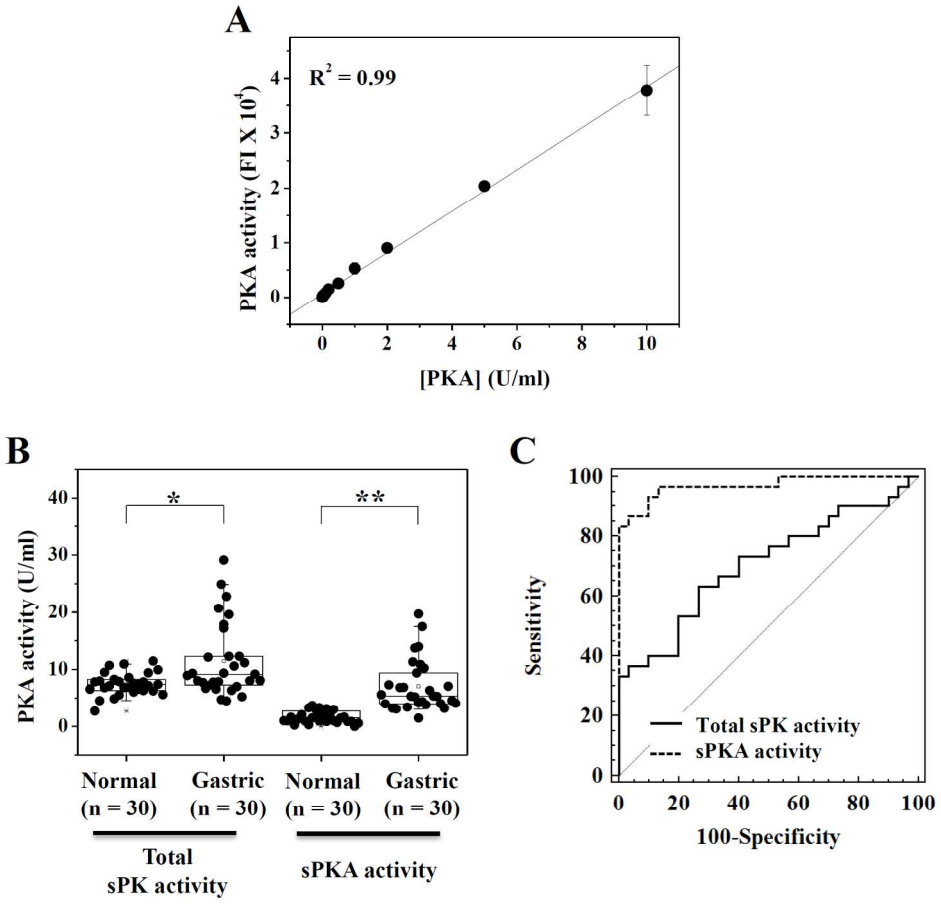
752x674mm (72 x 72 DPI)

Fig. 3. Sensitivity enhancement of on-chip sPKA activity assay by Triton X-100 and inhibition by PKI.



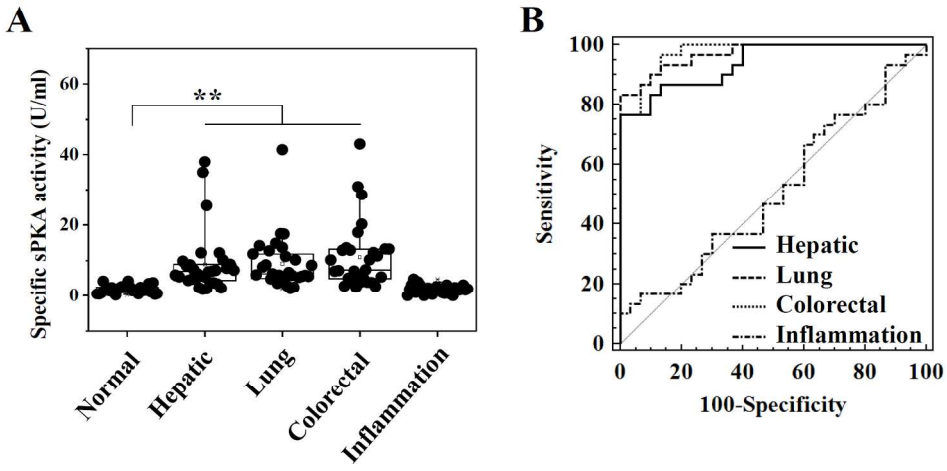
770x742mm (72 x 72 DPI)

Fig. 4. Suitability of on-chip activity assay for high throughput quantitation of sPKA activities in human sera.



764x770mm (72 x 72 DPI)

Fig. 5. Analysis of sPKA activity in human sera from normal individuals, three types of cancer patients, and individuals with inflammation.



770x504mm (72 x 72 DPI)

Peptide array-based serological protein kinase A activity assay and its application in cancer diagnosis

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Running title: serological PKA activity as a cancer biomarker

ABSTRACT

Protein kinase A (PKA) plays a crucial role in several biological processes; however, there is no assay with sufficient sensitivity and specificity to determine serological PKA (sPKA) activity. Here we present an on-chip activity assay that employs cysteine-modified kemptide arrays to determine specific sPKA activity in human sera that eliminates the potential contributions of other kinases with a protein kinase peptide inhibitor. The sensitivity of the on-chip sPKA activity assay was greatly enhanced by Triton X-100, with a 0.01 U/mL detection limit. sPKA activity was determined by subtracting nonspecific sPK activity from total sPK activity. Our assay provided greater sensitivity and specificity and more accurate area under the curve values for gastric cancer compared to the total sPK activity assay. sPKA activities in human sera from patients with hepatic (n = 30), gastric (n = 30), lung (n = 30), and colorectal (n = 30) cancers were significantly higher than those in controls (n = 30, $p < 10^{-4}$), but no significant difference in sPKA activities between normal and inflammation groups was observed. These results demonstrate that the on-chip assay accurately measures sPKA activity in human sera and that sPKA activity may be a potential biomarker for cancer diagnosis.

Keywords: serological PKA activity, cysteine-modified kemptide arrays, cancer biomarker, cancer diagnosis

INTRODUCTION

Protein kinase A (PKA) is one of the most important enzymes involved in post-translational modification and plays a crucial role in many biological processes, including gene induction, angiogenesis, and apoptosis.[1-3] PKA, including types I and II, is a predominantly intracellular tetrameric enzyme comprised of two regulatory and two common catalytic subunits; upon cyclic AMP (cAMP) binding, it dissociates into an R dimer and two free C subunits.[4, 5] The catalytic form of PKA (cPKA) is excreted into the extracellular space by different types of cancer cells, including lung, bladder, colon, and renal cancer cells.[4] Extracellular PKA activity is elevated in human sera from cancer patients compared with samples from normal individuals,[3, 5, 6] implying that serological PKA (sPKA) could be a useful cancer biomarker.

PKA activity has traditionally been measured using radioactive isotope-labeled ATP;[3-7] however, this method is laborious, time-consuming, and associated with radiological hazards.[8] Alternative nonradioactive methods based on fluorescence,[9-11] luminescence,[12] nanoparticles,[8, 13, 14] and quartz crystal microbalance[15] overcome these shortcomings. Fluorescence detection methods employ molecular probes such as Pro-Q Diamond dye[9] and biotinylated phosphate-specific ligands.[10, 11] Various types of nanoparticles, including gold,[8, 16] quantum dots,[13] and zirconium ion-immobilized magnetic nanoparticles[14] have also been used to improve PKA activity assay sensitivity. While useful, these methods are limited in quantifying PKA activity in mixed samples such as human sera because different kinases can phosphorylate substrates[17-19] and thus interfere with specific sPKA activity measurement in blood samples. Therefore, there is a need to develop highly sensitive, specific, and easily manipulatable assays for assessing sPKA activity.

In this study, we describe the use of cysteine-modified kemptide (C-kemptide) arrays for on-chip sPKA activity assays of human sera to yield quantitative, highly sensitive, specific, and reproducible results. Our method eliminates the possible contribution of other kinases by using a specific PKA peptide inhibitor (PKI)[20]. We used this assay to evaluate the utility of sPKA activity as a potential cancer biomarker and found that sPKA activities in human sera from patients with hepatic, gastric, lung, or colorectal cancer were much higher than those from controls. Our results indicate that the on-chip activity assay is suitable to determine sPKA activity in human sera to support a cancer diagnosis. Furthermore, this activity assay could be useful for assessing PKA-related human diseases.

EXPERIMENTAL SECTION

Chemical reagents

3-Aminopropyltrimethoxysilane and H89 were obtained from Sigma-Aldrich (St. Louis, MO). PKI and cPKA were purchased from Promega (Madison, WI) and Biaffin GmbH & Co KG (Kassel, Germany), respectively. N-[γ-maleimidobutyloxy]succinimide ester (GMBS) was obtained from Pierce (Rockford, IL). C-kemptide (C-G-G-L-R-R-A-S-L-G, free from on the N- and C-termini), which has three additional amino acids (cysteine, glycine, and glycine) on the N-terminus of kemptide (L-R-R-A-S-L-G), was synthesized by Peptron (Daejeon, Korea). Pro-Q Diamond phosphoprotein gel stain and destaining solution were purchased from Invitrogen (Carlsbad, CA). Polydimethylsiloxane (PDMS) solution was obtained from Sewang Hitech (Gimpo, Korea).

Serum samples

Human serum samples collected from normal individuals (n = 30), patients with hepatic (n = 30), gastric (n = 30), lung (n = 30), or colorectal (n = 30) cancer, and individuals with inflammation (n = 30, >3 μg/mL C-reactive protein [CRP]) were provided by the Kangwon National University Hospital Biobank (a member of the National Biobank Korea, Korea) and stored at -80°C until use. Experiments using human samples were performed with the approval of the local Institute's Ethics Committee for human subject research.

PDMS gasket fabrication

PDMS prepolymer solution was prepared by mixing 5 g PDMS base and 0.5 g curing agent until it became cloudy with bubbles. Next, degassing was performed for 30 min,

and 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 diameter were detached.

Well-type peptide array fabrication

Amine-modified glass slides were prepared as previously reported.[21] Briefly, glass slides (75 × 25 mm) were cleaned with an $H_2O_2/NH_4OH/H_2O$ (1:1:5, v/v) solution, immersed in 1.5% 3-aminopropyltrimethoxiysilane (v/v) for 2 h, and baked at 110°C. Well-type amine arrays were fabricated by mounting PDMS gaskets onto the amine-modified glass slides. Well-type peptide arrays were fabricated by sequential modification of amine arrays with 5 mM sulfo-GMBS in 50 mM sodium bicarbonate buffer (pH 7.0) and 10 μg/mL substrate peptide in phosphate buffer (8.1 mM Na_2HPO_4 and 1.2 mM KH_2PO_4, pH 7.4).

On-chip PKA activity assay using fluorescence-based peptide arrays

PKA activity assays were performed on well-type peptide arrays using a Pro-Q Diamond stain as described in Fig. 1. Peptide arrays were blocked with 1% BSA in TBS; 13.8 mM NaCl and 2 mM Tris-HCl, pH 7.4) containing 0.1% Tween-20 for 30 min at 37°C and sequentially washed with TBS containing 0.1% Tween-20 and milli-Q water. Next, 1 μL of reaction mixture containing activity assay buffer (50 mM Tris-HCl [pH 7.5], 0.5 mM $MgCl_2$, 0.01% Triton X-100, 0.5 mM ATP, and 0.2% human serum albumin) and diluted sera (20-fold) was applied to peptide arrays in the absence or presence of 1 μM PKI and incubated at 30°C for 90 min. Reaction mixtures containing various concentrations of cPKA were also applied to peptide arrays to create a standard

curve. One unit of cPKA was defined as the amount of enzyme that is required to incorporate 1 pmol of phosphate into the specific substrate peptide kemptide in 1 min at 30°C. Phosphorylated serine residues of peptide substrates were probed by incubation with Pro-Q Diamond stain at room temperature for 60 min. Arrays were washed twice with destaining solution for 15 min and twice with milli-Q water for 5 min each. The arrays were then scanned with a fluorescence scanner equipped with a 543-nm laser (ScanArray Express GX, Perkin Elmer, Waltham, MA), and the resulting fluorescence intensities (FIs) of array spots were used to determine PKA activities. The limit of detection (LOD) of PKA activity was calculated using the following equation: LOD = blank + S.D. × 3,

where S.D. is the standard deviation of the blank sample.

Determination of sPKA activity

For quantitative determination of PKA activity, we created a standard curve using the linear fit of the Origin program:

$F = cA + d$,

where F is the FI of the samples on the array surface, c and d are the slope and intercept of the linear fit of a standard curve, respectively, and A is the PKA activity. sPKA activities in serum samples (U/mL) were calculated from the difference between PK activity with and without PKI with the following equation.

$A_{sPKA} = A_{Tot} - A_{Non}$,

where A_{sPKA} is sPK activity, A_{Tot} is total sPKA activity in the absence of PKI, and A_{Non} is nonspecific sPK activity in the presence of PKI.

Data analysis

ScanArray Express software (Perkin Elmer) was used for FI quantification and data extraction. The Origin 6.0 software package (Origin Lab, Northampton, MA) was employed to perform *t* tests comparing two populations. A p value < 0.05 was considered statistically significant. Receiver operating characteristic (ROC) analyses were performed using MedCalc statistical software 11.4.4.0 (Mariakerke, Belgium) to calculate sensitivity, specificity, and area under the curve (AUC).

RESULTS AND DISCUSSION

Optimization of on-chip sPKA activity assay using fluorescence-based peptide arrays

Human serum contains various protein kinases in addition to PKA,[17-19] and these kinases could interfere with the specific measurement of sPKA activity in blood samples. To minimize the contribution of other kinases to serine phosphorylation of the substrate peptide, we designed an on-chip sPKA activity assay that employed the PKI, which specifically inhibits cPKA activity[20] (Figure 1). sPKA activity in human serum was determined by subtracting nonspecific sPK activity (in the presence of PKI) from the corresponding total sPK activity (in the absence of PKI).

The substrate peptide (C-kemptide) was prepared by modifying kemptide using three aminoacids (cysteine, glycine, glycine) on its N-terminus for oriented immobilization of the substrate peptide onto GMBS-modified amine arrays (Figure 1). We optimized the C-kemptide concentration by immobilizing various concentrations of the peptide ranging from 0.1 to 20 μg/mL in the well-type arrays. PKA activity, expressed as FI, increased in a C-kemptide concentration-dependent manner, with maximal effect at 10 μg/mL (Figure 2A). This suggests that C-kemptide can be used as a substrate for the PKA activity assay, ideally at 10 μg/mL.

Catalytic PKA has three conformation states according to the binding of ATP, Mg^{2+}, and a substrate or inhibitor that affects its activity change.[22] We determined the optimal concentrations of $MgCl_2$ and ATP by applying reaction mixtures containing the indicated concentrations of $MgCl_2$ and ATP to GMBS-modified well-type arrays. $MgCl_2$ elevated PKA activity in a dose-dependent manner, with maximal stimulation at 0.5 mM (Figure 2B). ATP also induced PKA activation in a dose-dependent manner, with saturation at 0.5 mM (Figure 2C). In addition, PKA activity increased in a time-

dependent manner (Figure 2D).

Sensitivity enhancement by Triton X-100 and characterization of the sPKA activity assay

Because Triton X-100 was reported to increase the sensitivity of a transglutaminase activity assay,[23] we tested whether the nonionic detergent could enhance the detection signal of the on-chip sPKA activity assay. Surprisingly, Triton X-100 increased PKA activity in a dose-dependent manner, with an evident increase at 0.001% and saturation at 0.01% (Figure 3A). We then studied the effect of Triton X-100 on PKA activity assay sensitivity using the indicated concentrations of cPKA in the presence or absence of Triton X-100 (Figure 3B). Triton X-100 significantly enhanced PKA activities at all concentrations, and the LOD of the PKA activity assay was dramatically improved from 1.45 to 0.01 U/mL by 0.01% Triton X-100, demonstrating that it sensitized the assay by at least 100-fold. The LOD of this assay (0.01 U/mL) is superior to those described in previous reports.[14, 15, 24] Enhanced sensitivity by Triton X-100 might be explained by increased accessibility of sPKA and/or cofactors to the substrate peptide on well-type arrays.

We then evaluated on-chip sPKA activity assay reproducibility using 150 human sera samples. Inter-array reproducibility was determined to be high by analyzing the same batch of reaction mixtures on different arrays; the average correlation coefficient was 0.990 (n = 3, coefficient of variability [CV] = 0.7%). Inter-spot reproducibility was determined by analyzing 20 replicate spots, and the average CV was 2.2% (n = 3). Taken together, these results confirm that the assay is highly reproducible.

We also investigated the inhibitory effect of PKI, known as a PKA-specific peptide inhibitor, on sPKA activity assay. As shown in Figure 3C, using 100 U/mL

cPKA, PKI inhibited PKA activity in a dose-dependent manner, with complete inhibition at 1.0 μM. The half-maximal inhibitory concentration (IC_{50}) of PKI for PKA activity was calculated to be 4.3 nM. These results indicate that 1 μM PKI is sufficient to inhibit kinase activity up to 100 U/mL cPKA and can be used for determining specific sPKA activities in human sera with the on-chip activity assay.

Suitability of on-chip activity assay for high-throughput quantification of sPKA activities in human sera

Human serum contains various kinases including PKA, protein kinase C, and AMP-activated protein kinase[17-19] that could interfere with the analysis of sPKA activity in human sera. To evaluate whether the on-chip activity assay is suitable for high-throughput quantification of sPKA activities in human blood samples, we determined sPKA activities in human sera from normal individuals (n = 30) and gastric cancer patients (n = 30) in the absence or presence of PKI. PKI was used to eliminate the possible contribution of other serine/threonine kinases. The sPKA activities were quantitatively measured using a standard curve created from FIs of cPKA (Figure 4A). PKA activity linearly increased from 0.01 to 10 U/mL (R^2 = 0.99). sPKA activities in human sera were determined by subtracting nonspecific sPK activities (in the presence of PKI) from the corresponding total sPK activities (in the absence of PKI). As shown in Figure 4B, gastric cancer patients exhibited higher total sPK activities than normal individuals ($p < 0.01$). Surprisingly, the sPKA activity assay revealed a much greater difference between the two groups ($p < 10^{-4}$). These results demonstrate that total sPK activities were influenced by other kinases besides PKA and that the sPKA activity assay is more accurate for analyzing sPKA activity in human blood samples.

We then performed ROC analysis for gastric cancer patients to evaluate the

utility of sPKA activity as a cancer biomarker (Figure 4C). Compared to the total sPK assay, the specific sPKA activity assay was much more sensitive (63.3% versus 96.7%) and specific (73.3% versus 86.7%), and the AUC value was greater for the specific test (0.706 versus 0.970). These findings demonstrate that the on-chip activity assay is suitable for the rapid determination of sPKA activities in human sera and sPKA activity might be a cancer biomarker.

Evaluation of sPKA activity as a cancer biomarker

To further study whether sPKA activity can be used as a serodiagnostic biomarker for cancer, we compared sPKA activities in human sera from normal individuals (n = 30) and patients with hepatic (n = 30), lung (n = 30), and colorectal (n = 30) cancers (Figure 5A). The mean sPKA activities of the normal and patient groups with hepatic, lung, and colorectal cancer were 1.78 ± 1.09, 8.92 ± 8.72, 9.06 ± 7.50, and 10.94 ± 9.34 U/mL, respectively, demonstrating that sPKA activities were significantly higher ($p < 10^{-4}$) in the three cancer patient groups compared to healthy controls.

We performed ROC analyses for the three types of cancers to further evaluate the utility of sPKA activity as a potential cancer biomarker (Figure 5B). The respective sensitivities and specificities of sPKA activity for the three types of cancers were hepatic (83.3% and 90.0%), lung (90.0% and 90.0%), and colorectal (90.0% and 90.0%), with high AUC values of 0.939 (95% confidence interval [CI], 0.85–0.98), 0.970 (95% CI, 0.89–0.99), and 0.974 (95% CI, 0.90–0.99), respectively (Table 1). Additionally, the sPKA activities of all cancer patients (n = 120) including those with gastric cancer showed 90.0% sensitivity and 90.0% specificity at the cutoff value of 3.5 U/mL, with an AUC value of 0.966 (95% CI, 0.92–0.98). These results further demonstrate that specific sPKA activity is a potential biomarker for the diagnosis of cancer.

sPKA activity is not an inflammation biomarker

Various serological biomarkers have been used for diagnosing cancer, including α-fetoprotein; prostate-specific antigen; carcinoembryonic antigen; and CA 15-3, 19-9, and 125.[7, 25, 26] However, biomarkers based on antigen determination lack adequate sensitivity and specificity. To investigate whether sPKA activity is a biomarker specific to cancer, we analyzed sPKA activity in human sera from normal (n = 30) and inflammation (n = 30) groups. The inflammation group included samples from individuals with >3 μg/mL CRP in sera. CRP is an acute-phase protein that is widely used as an inflammation biomarker.[27, 28] No significant difference in sPKA activities between normal and patient groups was observed (Figure 5A). The ROC analysis of the inflammation group showed poor sensitivity (55.2%) and specificity (53.3%), with a low AUC value of 0.530 compared to the four cancer patient groups (Figure 5B and Table 1). These results demonstrate that sPKA activity is not an informative biomarker for inflammation, providing additional evidence that sPKA activity is a useful cancer biomarker.

CONCLUSION

In this study, we described an on-chip activity assay using fluorescence-based substrate peptide arrays to quantitate specific sPKA activity in human sera and investigated the utility of this measurement as a serological biomarker of cancer. This on-chip assay employed C-kemptide arrays fabricated using PDMS gaskets and is highly sensitive, quantitative, high-throughput, reproducible, and cost-effective. Samples from patients with hepatic, gastric, lung, or colorectal cancer showed significantly higher sPKA activities than those collected from normal individuals. The on-chip sPKA activity assay showed high sensitivity, specificity, and AUC values for cancer patients, but no significant difference in sPKA activities between the control and inflammation groups was observed. Collectively, our findings indicate that this on-chip sPKA activity assay is appropriate for the high-throughput determination of sPKA activity in human sera and sPKA activity might be useful as a cancer biomarker.

ACKNOWLEDGEMENTS

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Competing Interests

The authors have declared that no competing interest exists.

Table 1. ROC analysis data of sPKA activities for four types of cancers and inflammation in human sera.

		Sensitivity (%)	Specificity (%)	AUC
Cancer	Hepatic (n = 30)	83.3	90.0	0.939
	Gastric (n = 30)	96.7	86.7	0.970
	Lung (n = 30)	90.0	90.0	0.970
	Colorectal (n = 30)	90.0	90.0	0.974
	Total (n = 120)	90.0	90.0	0.966
Inflammation (n = 30)		55.2	53.3	0.530

REFERENCES

1. S. Namkoong, S. J. Lee, C. K. Kim, Y. M. Kim, H. T. Chung, H. Lee, J. A. Han, K. S. Ha and Y. G. Kwon, *Exp Mol Med*, 2005, **37**, 588-600.
2. Y. S. Cho-Chung, S. Pepe, T. Clair, A. Budillon and M. Nesterova, *Crit Rev Oncol Hematol*, 1995, **21**, 33-61.
3. H. Wang, M. Li, W. Lin, W. Wang, Z. Zhang, E. R. Rayburn, J. Lu, D. Chen, X. Yue, F. Shen, F. Jiang, J. He, W. Wei, X. Zeng and R. Zhang, *Cancer Epidemiol Biomarkers Prev*, 2007, **16**, 789-795.
4. Y. S. Cho, Y. G. Park, Y. N. Lee, M. K. Kim, S. Bates, L. Tan and Y. S. Cho-Chung, *Proc Natl Acad Sci U S A*, 2000, **97**, 835-840.
5. M. E. Cvijic, T. Kita, W. Shih, R. S. DiPaola and K. V. Chin, *Clin Cancer Res*, 2000, **6**, 2309-2317.
6. T. Kita, J. Goydos, E. Reitman, R. Ravatn, Y. Lin, W. C. Shih, Y. Kikuchi and K. V. Chin, *Cancer Lett*, 2004, **208**, 187-191.
7. M. V. Nesterova, N. Johnson, C. Cheadle, S. E. Bates, S. Mani, C. A. Stratakis, I. U. Khan, R. K. Gupta and Y. S. Cho-Chung, *Cancer Res*, 2006, **66**, 8971-8974.
8. T. Li, X. Liu, D. Liu and Z. Wang, *Anal Chem*, 2013, **85**, 7033-7037.
9. K. Martin, T. H. Steinberg, L. A. Cooley, K. R. Gee, J. M. Beechem and W. F. Patton, *Proteomics*, 2003, **3**, 1244-1255.
10. X. Han and Y. Katayama, *Methods Mol Biol*, 2010, **669**, 183-194.
11. K. Inamori, M. Kyo, K. Matsukawa, Y. Inoue, T. Sonoda, K. Tatematsu, K. Tanizawa, T. Mori and Y. Katayama, *Anal Chem*, 2008, **80**, 643-650.
12. M. Kasari, P. Padrik, A. Vaasa, K. Saar, K. Leppik, J. Soplepmann and A. Uri, *Anal Biochem*, 2012, **422**, 79-88.
13. X. Xu, X. Liu, Z. Nie, Y. Pan, M. Guo and S. Yao, *Anal Chem*, 2011, **83**, 52-59.
14. P. Tan, C. Lei, X. Liu, M. Qing, Z. Nie, M. Guo, Y. Huang and S. Yao, *Anal Chim Acta*, 2013, **780**, 89-94.
15. X. Xu, J. Zhou, X. Liu, Z. Nie, M. Qing, M. Guo and S. Yao, *Anal Chem*, 2012, **84**,

4746-4753.

16. T. Li, D. Liu and Z. Wang, *Anal Chem*, 2010, **82**, 3067-3072.

17. N. L. Anderson, M. Polanski, R. Pieper, T. Gatlin, R. S. Tirumalai, T. P. Conrads, T. D. Veenstra, J. N. Adkins, J. G. Pounds, R. Fagan and A. Lobley, *Mol Cell Proteomics*, 2004, **3**, 311-326.

18. J. H. Kang, T. Mori, H. Kitazaki, T. Niidome, K. Takayama, Y. Nakanishi and Y. Katayama, *Anticancer Res*, 2013, **33**, 485-488.

19. E. Malvoisin, J. M. Livrozet, I. El Hajji-Ridah, D. Makloufi and N. Vincent, *J Immunol Methods*, 2009, **351**, 24-29.

20. G. D. Dalton and W. L. Dewey, *Neuropeptides*, 2006, **40**, 23-34.

21. J. W. Jung, S. H. Jung, J. O. Yoo, I. B. Suh, Y. M. Kim and K. S. Ha, *Biosens Bioelectron*, 2009, **24**, 1469-1473.

22. A. Y. Kovalevsky, H. Johnson, B. L. Hanson, M. J. Waltman, S. Z. Fisher, S. Taylor and P. Langan, *Acta Crystallogr D Biol Crystallogr*, 2012, **68**, 854-860.

23. M. H. Kwon, J. W. Jung, S. H. Jung, J. Y. Park, Y. M. Kim and K. S. Ha, *Mol Cells*, 2009, **27**, 337-343.

24. J. Zhou, X. Xu, X. Liu, H. Li, Z. Nie, M. Qing, Y. Huang and S. Yao, *Biosens Bioelectron*, 2014, **53**, 295-300.

25. M. Nesterova, N. Johnson, C. Cheadle and Y. S. Cho-Chung, *Biochim Biophys Acta*, 2006, **1762**, 398-403.

26. D. H. Kong, J. W. Jung, K. Na, S. K. Jeong, Y. K. Paik, S. H. Jung, I. B. Suh, Y. M. Kim and K. S. Ha, *Anal Chim Acta*, 2012, **718**, 92-98.

27. H. Y. Jeon, D. H. Kong, S. H. Kim, I. B. Suh, E. T. Han, Y. M. Kim and K. S. Ha, *Biochip J*, 2015, **9**, 35-43

28. B. Clyne and J. S. Olshaker, *J Emerg Med*, 1999, **17**, 1019-1025.

Figure legends

Figure 1. Schematic diagram of the sPKA activity assay for human sera using fluorescence-based peptide arrays. Cys, cysteine; GMBS, N-[γ-maleimidobutyryloxy]sulfosuccinimide ester; Ser, serine.

Figure 2. On-chip sPKA activity assay optimization. (A) Determination of the optimal C-kemptide concentration for fabricating well-type peptide arrays. (B-C) Reaction mixtures were prepared by mixing the indicated concentrations of $MgCl_2$ (B) and ATP (C) with 100 U/mL human cPKA in the reaction buffer and applied to well-type peptide arrays fabricated using 10 μg/mL C-kemptide. (D) A reaction mixture with a 1-μL volume containing 0.5 mM $MgCl_2$, 0.5 mM ATP, and 100 U/mL human cPKA was applied to the peptide arrays and incubated for the indicated time. PKA activity was determined by measuring the FIs of array spots as described in the Experimental Section. The results are expressed as the mean ± S.D. of three independent experiments.

Figure 3. Sensitivity enhancement of the on-chip sPKA activity assay by Triton X-100 and inhibition by PKI. (A) Dose-dependent increases in PKA activity with Triton X-100. Reaction mixtures containing the indicated concentrations of Triton X-100 and 100 U/mL human cPKA were loaded onto peptide arrays and incubated at 30°C for 90 min. PKA activities were determined as described in the Experimental Section. (B) Sensitivity enhancement of the on-chip PKA activity assay by Triton X-100. Inset, LOD. (C) Dose-dependent inhibition of PKA activity by PKI. PKA activities are expressed as % of the control. The results are expressed as the mean ± S.D. of three independent experiments.

Figure 4. Suitability of the on-chip activity assay for high-throughput quantification of sPKA activities in human sera. (A) The standard curve ($r^2 = 0.99$). (B-C) Human sera from normal individuals (n = 30) and gastric cancer patients (n = 30) were diluted 20-fold in the absence (total sPK activity) or presence (nonspecific sPK activity) of 1 μM PKI, and reaction mixtures containing diluted human sera were applied to peptide arrays. The specific sPKA activities of 60 serum samples were determined using a standard curve as described in the Experimental Section. (B) Distribution of total sPK and specific sPKA activities. The boxes represent the upper and lower quartiles of specific sPKA activities, and the horizontal lines in each box indicate the median values. *$p < 0.01$, **$p < 10^{-4}$. (C) ROC plots of total and specific sPKA activities.

Figure 5. Analysis of sPKA activity in human sera from normal subjects, three types of cancer patients, and individuals with inflammation. Reaction mixtures containing human sera (20-fold diluted) from normal subjects (n = 30); patients with hepatic (n = 30), lung (n = 30), or colorectal (n = 30) cancer; and individuals with inflammation (n = 30) were applied to the peptide arrays. The specific sPKA activities of serum samples were determined using a standard curve as described in the Experimental Section. (A) Distributions of specific sPKA activities. **$p < 10^{-4}$ versus normal. (B) ROC plots of specific sPKA activities.