Bioluminescent aptamer-based solid-phase microassay to detect lung tumor cells in plasma

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Abstract

Two high-affinity DNA aptamers for lung tumor cells were applied as biospecific elements in bioluminescent assay of patient blood. The oligonucleotide complementary to the 5' end of both aptamers carrying either biotin or Ca^{2+}-regulated photoprotein obelin was used to form a sandwich-type analytical complex on the surfaces of magnetic streptavidin-activated microspheric particles. Clinical blood samples from cases of morphologically confirmed lung cancer and control samples were analyzed applying the developed assay. From the receiver-operator curve (ROC) analysis, the chosen threshold value as clinical decision limit offers

sensitivity of 91.5% and specificity of 75% ($p<0.001$). The area under ROC curve (AUC) with the value of 0.901 distinguishes well between the two groups under investigation.

1. Introduction

Analytical technologies, both *in vitro* and *in vivo*, based on light-emitting (bioluminescent) proteins – luciferases and photoproteins as effective reporters, are gaining more and more popularity. This is due to availability of the enzymes, their non-toxicity, impressive simplicity of the reactions, and high sensitivity of the assay which they provide [1, 2]. The combination of luciferase with a biospecific recognition molecule (immunoglobulin, oligonucleotide, hapten, etc.) offers a sensitive and selective tool (bioluminescent labels) for molecular binding assay.

Aptamers are single-chain DNA or RNA oligonucleotides (20-80 bases long) with a unique spatial structure formed by the local complementary duplexes, stacking interactions, hydrogen bonds net etc. The conventional selection procedure provides to aptamers with a high affinity to the desired target from randomly synthesized library [3]. Aptamers as biospecific recognition molecules successfully compete with antibodies because they are easily synthesized chemically, and can be selected for almost any target, including that of low molecular weight, toxic and non-immunogenic. Owing to these advantages, aptamers are increasingly being used as biospecific elements in a number of biomedical analytical systems [4]. The targets for aptamer selection can be not only biochemically pure entities but complex biological systems like cultural cells, e.g. at cancer investigations [5-9] and tissues. Recently a group of ten DNA aptamers to postoperative lung adenocarcinoma was selected [10]. All of them demonstrated high affinity to the tumor tissue elements (mostly cells) and the binding absence with healthy lung tissue and blood cells. Fluorescently labeled aptamers from this group – LC-17 and LC-18, were successfully applied for detection of blood circulating tumor cells in patients with lung adenocarcinoma by confocal fluorescent microscopy [10] and for

histological examinations of lung adenocarcinoma [11]. The aim of our research was to develop the solid-phase assay based on this couple of aptamers (LC-17 and LC-18) as sensing elements and the recombinant Ca^{2+}-regulated photoprotein obelin as a high sensitive bioluminescent reporter to detect lung tumor elements circulating in blood. The 98 clinical blood samples (from patients with lung cancer or other lung diseases and healthy donors) were investigated using the assay. The main analytical characteristics of the proposed method and its prospects were evaluated.

2. Materials and methods

The highly purified Ca^{2+}-regulated photoprotein obelin with a unique cysteine residue (Obe D12C) was obtained as described previously [12]. Aptamers LC-17, and LC-18 (Fig S1) were selected to postoperative lung adenocarcinoma using cell-SELEX technique as described in [10]. The aptamers solutions were heated at 95° C for 5 min and cooled for 5 min on ice before use.

Oligonucleotides 5'-CGTGGTTACAGTCAGAGGAG-3'-Bio (olig1) and 5'-CGTGGTTACAGTCAGAGGAG-3'-$(CH_2)_6$-NH_2 (olig2) were synthesized by Biosan (Russia).

All other reagents were of highest quality grade.

Solid-phase bioluminescent assay was carried out using magnetic particles with covalently immobilized streptavidin (Promega, USA).

Blood samples of 56 patients were obtained from the Krasnoyarsk regional clinical oncology center named after A.I. Kryzhanovsky. Blood samples of patients were collected prior to surgery for tumor resection. After surgery, the diagnosis of lung cancer was histologically confirmed for 47 patients; 3 cases were lung metastases of the other cancer types. Together these 50 cases formed the experimental group. The control group contained 6 samples from patients with the other diagnoses and 42 samples from healthy volunteers.

The study was approved by the local Ethics Committee of Krasnoyarsk regional clinical oncology center named after A.I. Kryzhanovsky. It was carried out in accordance with the ethical standards as stated in the Helsinki Declaration. The informed consent was obtained from all the patients under study.

2.1. Blood sample preparation

Blood samples with anticoagulant (ethylenediaminetetraacetic acid, EDTA) were centrifuged at 3000 rpm for 20 min to obtain plasma. Plasma samples were used for analysis within 24 h after collection of blood and were not frozen. Before testing, masking yeast RNA (1 ng μL^{-1}) was added to the plasma sample and incubated with shaking for 30 min at room temperature.

2.2. The chemical conjugate obelin-olig2 was synthesized as described earlier [13]. Briefly, olig2 was incubated with a 50-fold molar excess of succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) (Fluka, Switzerland) for 2 h at room temperature in 0.1 mM $NaHCO_3$, purified by chromatography and then incubated with a 3-fold molar excess of ObeD12C in 20 mM, Tris-HCl, pH 7.0, 5 mM EDTA overnight at 4° C. The conjugate was purified by anion-exchange chromatography on Mono Q column (GE Healthcare, UK), equilibrated with 20 mM Tris-HCl, pH 7.0, 5 mM EDTA in NaCl concentration gradient (0−1 M).

2.3. Conjugate obelin-olig2 detection limit

Serial conjugate dilutions in 20 mM Tris-HCl pH 7.0, 5 mM EDTA, 0.1 mg mL^{-1} BSA (100 µL) were placed into microtiter wells and bioluminescence was measured with a Mithras LB 940 plate luminometer (Berthold, Germany) immediately after the rapid injection of 100 µL of $CaCl_2$ solution (0.1 M, in 0.1 M Tris-HCl pH 8.8). The signal was integrated for 5 s. Detection limit was calculated as a sample concentration with a signal-to-background ratio of 2 (three replicates).

2.4. Cells suspensions preparation

The samples of cells suspensions were prepared from 1 g of lung tissue. Cancer material was washed with Dulbecco's phosphate-buffered saline (DPBS), minced into small pieces and then gently pipetted with DPBS to remove cells clusters and obtain a homogeneous suspension. Cell suspension was filtered through 70 μL filters, centrifuged at 3000g for 5 min and the cells pellet washed three times with DPBS followed by centrifugation. Final pellet was resuspended in 100 mM PBS (phosphate-buffered saline, pH 7.5) and used for the assay.

2.5. Bioluminescent solid-phase assay

The 240 μL plasma sample or postoperative lung cells suspension or PBS (control) was incubated with equimolar mixture of 60 μL LC-17 aptamer and olig1 (each final concentration of 250 nM) in 20 mM Tris-HCl, pH 7.0, 5 mM EDTA, and magnetic particles (30 μg per analysis), for 30 min with shaking, at room temperature. After incubation, the particles were washed three times with solution of 20 mM Tris-HCl, pH 7.0, 5 mM EDTA (hereinafter – the buffer) fixing the particles on the tube wall by magnet and sucking off the supernatant.

The 60 μL LC-18 aptamer (250 nM in 20 mM Tris-HCl, pH 7.0, 5 mM EDTA) was added to the particles, incubated and washed in the same way. Next, a conjugate obelin-olig2, was added into the tube (1.3 μg mL^{-1} in 20 mM Tris-HCl, pH 7.0, 5 mM EDTA), incubated and washed similarly.

The particles suspended in 60 μL of the buffer were transferred into the wells of an opaque 96-well microplate (Costar, USA) and the bioluminescent signal was measured immediately after the rapid injection of 100 μL of $CaCl_2$ solution as described above.

The averaged signal, received from control samples (n=2) was taken as the background and subtracted from the ones from working wells.

2.6. Statistical analysis

The collected data were statistically analyzed using Statistica software, version 12.0 (StatSoft, Russia) and MedCalc Software for Windows, version 18.2.1 (MedCalc Software, Ostend, Belgium).

The bioluminescent signals of sample from patients and controls were compared by Mann-Whitney U-test and assessed using the area under a receiver operating characteristic (AUC) curve. The cut-off value was determined by the optimal Youden's index (sensitivity + specificity -1) and used to calculate sensitivity and specificity.

The results of the assay obtained for the two groups of patients with different cancer types were compared by Mann-Whitney U-test. Those obtained from patients with different cancer stages were compared by Kruskal-Wallis test. The p-value <0.05 was considered as significant.

3. Results

3.1. Study design

In the present research, we developed a solid-phase sandwich type assay of lung cancer elements (mostly cells) in plasma based on DNA aptamers LC-17 and LC-18 selected to the postoperative lung cancer tissues as biospecific elements and Ca^{2+}-regulated photoprotein obelin as a highly sensitive bioluminescent reporter (Fig. 1). LC-17 and LC-18 possess high affinity to post operative lung tissue with k_d= 14 nM, and 38 nM correspondingly [10], bound different ligands and were already successfully applied as reporter for characterization of histological structure of lung adenocarcinoma [11].

Fig. 1. Scheme for the solid-phase bioluminescent assay.

Aptamer molecules are arranged similarly: they include constant fragments at 3' and 5' ends (20 n.b. each), whereas their central parts differ in sequences and frequently form spatial

structures, responsible for target binding. An oligonucleotide complementary to the 5' end of both aptamers was synthesized in two variants: the first one contained biotin at the 5' end (olig1), the second – amino group connected through hexamethylene spacer (olig2). Olig1 was immobilized on the magnetic streptavidin-covered microparticles, and the first aptamer LC-17 was attached to it due to 5' end complementarity. The resulting microparticle surface captured the target due to LC-17 affinity.

The second aptamer LC-18 forms a sandwich-type structure with the target. The resulting complex is detected with the conjugate obelin-olig2, serving as a bioluminescent label. Ca^{2+}-regulated photoprotein obelin is a stable noncovalent complex of single-chain (22.2 kDa) polypeptide and 2-hydroperoxycoelenterasine. Its bioluminescence as a flash of blue light (λ_{max}= 480 nm) is triggered by the addition of Ca^{2+}. As far as the protein takes part in the reaction directly the dependence of bioluminescence on protein concentration is always linear. Figure 2A shows the dependence of olig2-obelin conjugate bioluminescence on its amount. The detection limit of the conjugate is 20.8 ± 1.5 amol confirming it as a high sensitive label for binding assay.

Fig. 2. (A) Bioluminescence as a function on olig2-obelin conjugate amount. Each point is an average ± 1 standard deviation (n = 3). Some error bars are obstructed by the symbols. Signal from empty buffer is shown in red. (B) Model assay of two samples prepared from cancer (gray columns) and healthy (white column) lung tissues. r.l.u. - relative light units.

To test the approach, model solid-phase microassay of sandwich type was carried out using the samples of postoperative cancer and healthy lung tissues as targets (Fig. 2B). The samples were prepared as those used for the aptamers' selex [10]. Bioluminescent signal depends on the dilutions of cancer samples whereas the signal from the sample of healthy lung tissue remains at a constant low level.

3.2. Clinical samples analysis

Totally 98 plasma samples were analyzed by the developed assay. Samples from 56 patients (49 men and 7 women aged 34 to 86 years) were collected prior to surgery for tumor resection. Note, that proportion of men/women cases of lung cancer in our sampling corresponds with the one taking place in Krasnoyarsk region: 78.6 cases per 100 thousand males vs 15 per 100 thousand females [14]. Control samples contained 42 healthy in terms of lung cancer volunteers (14 men and 28 women aged 20 to 67 years). Diagnosis of lung cancer was histologically proved for the 47 patients: adenocarcinoma (22); squamous cell carcinoma (23); small cell and glandular squamous cell carcinoma (1 each). Finally, control group consisted of 48 samples (42 healthy volunteers plus 6 patients with non-cancer lung diseases).

The results of the bioluminescent aptamer-based assay in plazma samples from patients with lung cancer and control group are shown in Figure 3. Elevated signals levels in lung cancer patients were statistically significant ($p<0.001$). From the receiver-operator curve (ROC) analysis, the chosen threshold value as clinical decision limit offers sensitivity of 92% and specificity of 88% (Fig. 3A). The area under the ROC curve (AUC) value of 0.928 distinguishes well between the two groups under investigation (Fig. 3B).

Fig. 3. (A) The levels of cancer-associated target in histologically confirmed lung cancer patients and control group. Blue circuses present the results of the samples from patients with: 1,4 –pneumosclerosis; 2 – Hodgkin lymphoma; 3,5 – tuberculosis; 6 – chondroid hamartoma; 7-9 – lung metastases of other cancer types. (B) The ROC curve analysis for evaluating the proposed assay.

Bioluminescent analysis of plazma samples and histological investigation were carried out independently. Among the samples under investigation, there were several cases with

histologically unconfirmed lung cancer, namely: chondroid hamartoma (1), Hodgkin lymphoma (1), tuberculosis (2), pneumosclerosis (2), lung metastases of other cancer types (3). All these samples were analyzed with the developed bioluminescent assay as well (Fig. 3A). All the three cases of lung metastases of other cancer types gave undoubtedly positive results (Fig. 3A). The case of chondroid hamartoma gave a negative result, whereas the results on Hodgkin lymphoma, tuberculosis and pneumosclerosis were doubtful, but the amount of all these cases was not enough for interpretation. The possibility exists that the development of these non-cancer diseases causes similar targets in blood that are characteristic of lung carcinoma as well. This question is of great importance and needs further elucidation. For example, as it is described early [15] circulating tumor cells were found in blood of patients with chronic obstructive pulmonary disease and in these patients was diagnosed lung oncology after 1-4 years.

In our investigation, there were cases with: the first stage of cancer (cancer is found in the lung, but not spread outside the lung) – 10, the second – 12, the third – 18, and the fourth (cancer has spread to lung, into the area around the lungs, or to distant organs) – 7. The question was whether the proposed method of analysis could detect the different stages of cancer? The signals from samples with different stages were compared using Kruskal-Wallis test (Fig. 4A). As one may see there were no significant differences between the groups ($p>0.05$). Of note is a good result on the early stage detection: the assay is able to reveal 8 of 10 cases of the first cancer stage and 11 of 12 cases of the second stage. As to the late cancer stages, the assay detected 17 of 18 cases of the third stage and all the cases of the forth one.

Fig. 4. Bioluminescent signals from samples of patients with different stages (A) and type (B) of lung cancer. The line indicates the threshold value. Samples 1-6 show the results from the cases with histologically unconfirmed cancer lung cancer (see Fig. 3A). Blue line shows the threshold value (see Fig. 3A).

As we have noted above, the cases of adenocarcinoma (22) and squamous cell carcinoma (23) were prevailing in patients. It was found (Fig. 4B) that the developed assay could not differentiate the lung cancer type ($p>0.05$, Mann-Whitney U-test).

4. Discussion

Thus, the results of our research demonstrated a good potential of the developed bioluminescent assay based on two DNA aptamers with high affinity to lung cancer-associated targets: it provides differentiation between lung cancer and healthy cases with a high sensitivity and specificity. Previously suitability of fluorescently labeled aptamers LC-17 and LC-18 for the aptahistochemical staining of both tumor tissue and circulating tumor cells was shown by G. Zamai and co-authors [11]. To detect stained tissues or cells laser-scanning fluorescent microscopy or flow cytometry were applied that were expensive and requires highly qualified staff. Besides, these approaches need complex procedure of sample preparation. Presented here analytical approach based on the same aptamers affinity and bioluminescent reporter high sensitivity looks much simpler and cheaper.

Application of the other aptamers from the group selected to lung cancer may help to improve the specificity of the analysis aiming to exclude the cases of the other lung diseases. It is proposed that aptamers selected to the tissue are able to detect not only circulation of tumor cells but cellular debris, as well as cells forming the tumor microenvironment, involved into tumorigenesis [16]. That is why we suppose that our method allows detecting not only cancer cells, but also the other elements associated with the disease. The composition and dynamics of such elements is known to significantly differ in patients even with the same diagnosis [17] and therefore the use of a family of aptamers specific to cancer associated targets can be promising. Similar approach was developed by Smith and co-authors [18, 19], where target cells were detected using a panel of specific aptamers immobilized on the two

kinds of nanoparticles: one magnetic (to collect and enrich cancer cells) and the fluorescent one (to detect the cell using confocal microscope or fluorescent plate reader).

Design of the method proposed here makes possible application of other aptamers for simultaneous detection of several targets as far as it includes a set of the universal connecting labeled oligonucleotides to form the analytical complex on the microspheric particles. Since all aptamers contain the same technological sequences at the 3 'and 5' ends, the transition to multiplex variant of the analysis does not require the development of new labels or any changes in the analysis scheme. As opposed to the fluorescent, the bioluminescent signal of the obelin reporter is bright (due to high quantum yield of the reaction), with a high signal-to-noise ratio providing high sensitivity of the target detection. Simple obelin bioluminescence triggering and its fast signal in combination with microplate format provide the high throughput of the assay.

Thus, our studies demonstrate the potential of the bioluminescent assay on the base of aptamers as sensing elements for lung cancer detection and monitoring as well as future research direction.

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References

[1] Z. Yao, B.S. Zhang, J.A. Prescher, Advances in bioluminescence imaging: new probes from old recipes, Curr Opin Chem Biol. 45 (2018) 148–156.

[2] L.A. Frank, V.V. Krasitskaya, Application of enzyme bioluminescence for medical diagnostics, Adv Biochem Eng Biotechnol. 144 (2014) 175–197.

[3] A. Ozer, J.M. Pagano, J.T. Lis, New technologies provide quantum changes in the scale, speed, and success of SELEX methods and aptamer characterization, Mol Ther Nucleic Acids 3 (2014) e183.

[4] W. Zhou, P.-JJ. Huang, J. Ding, J. Liu, Aptamer-based biosensors for biomedical diagnostics, Analyst 139 (2014) 2627–2640.

[5] K.N. Morris, K.B. Jensen, C.M. Julin, M. Weil, L. Gold, High affinity ligands from *in vitro* selection: complex targets, Proc Natl Acad Sci USA 95 (1998) 2902–2907.

[6] D.A. Daniels, H. Chen, B.J. Hicke, K.M. Swiderek, L. Gold, A tenascin-C aptamer identified by tumor cell SELEX: Systematic evolution of ligands by exponential enrichment. Proc Natl Acad Sci USA 100 (2003) 15416–15421.

[7] H.W. Chen, C.D. Medley, K. Sefah, D. Shangguan, Z. Tang, L. Meng, J.E. Smith, W. Tan, Molecular recognition of small-cell lung cancer cells using aptamers, Chem Med Chem. 3 (2008) 991–1001.

[8] J.K. Herr, J.E. Smith, C.D. Medley, D. Shangguan, W. Tan, Aptamer-conjugated nanoparticles for selective collection and detection of cancer cells, Anal Chem. 78 (2006) 2918–2924.

[9] D. Shangguan, Y. Li, Z. Tang, Z.C. Cao, H.W. Chen, P. Mallikaratchy, K. Sefah, C.J. Yang, W. Tan, Aptamers evolved from live cells as effective molecular probes for cancer study, Proc Natl Acad Sci USA 103 (2006) 11838–11843.

[10] G.S. Zamay, O.S. Kolovskaya, T.N. Zamay, Y.E. Glazyrin, A.V. Krat, O. Zubkova, E. Spivak, M. Wehbe, A. Gargaun, D. Muharemagic, M. Komarova, V. Grigorieva, A. Savchenko, A.A. Modestov, M.V. Berezovski, A.S. Zamay, Aptamers selected to postoperative lung adenocarcinoma detect circulating tumor cells in human blood. Mol Ther. 23 (2015) 1486–1496.

[11] G.S. Zamay, T.I. Ivanchenko, T.N. Zamay, V.L. Grigorieva, Y.E. Glazyrin, O.S. Kolovskaya, I.V. Garanzha, A.A. Barinov, A.V. Krat, G.G. Mironov, A. Gargaun, D.V.

Veprintsev, S.S. Bekuzarov, A.K. Kirichenko, R.A. Zukov, M.M. Petrova, A.A. Modestov, M.V. Berezovski, A.S. Zamay, DNA Aptamers for the characterization of histological structure of lung adenocarcinoma, Mol Ther Nucleic Acids 6 (2017) 150–162.

[12] V.V. Krasitskaya, L.P. Burakova, A.A. Komarova, E.E. Bashmakova, L.A. Frank, Mutants of Ca^{2+}-regulated photoprotein obelin for site-specific conjugation. Photochem Photobiol. 93 (2017) 553–557.

[13] M.A. Vorobjeva, V.V. Krasitskaya, A.A. Fokina, V.V. Timoshenko, G.A. Nevinsky, A.G. Venyaminova, L.A. Frank, RNA aptamer against autoantibodies associated with multiple sclerosis and bioluminescent detection probe on its basis. Anal Chem. 86 (2014) 2590–2594.

[14] A.A. Modestov, I.P. Safontsev, R.A. Zukov, E.V. Slepov, M.P. Klimenok, E.N.Gaas, Cancer incident in the Krasnoyarsk krai. Russian Journal of Oncology, 21, No. 1 (2016) 76–80.

[15] M. Ilie, V. Hofman, E. Long-Mira, E. Selva, J-M. Vignaud, B. Padovani, "Sentinel" circulating tumor cells allow early diagnosis of lung cancer in patients with chronic obstructive pulmonary disease. PLoS ONE. 9 (2014) e111597.

[16] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation. Cell. 144 (2011) 646–674.

[17] J. Lehtiö, L. Petris, Lung cancer proteomics, clinical and technological considerations. J Proteomics 73 (2010) 1851–1863.

[18] J.E. Smith, C.D. Medley, Z. Tang, D. Shangguan, C. Lofton, W. Tan, Aptamer-conjugated nanoparticles for the collection and detection of multiple cancer cells. Anal Chem. 79 (2007) 3075–3082.

[19] C.D. Medley, S. Bamrungsap, W. Tan, J.E. Smith, Aptamer-conjugated nanoparticles for cancer cell detection, Anal Chem. 83 (2011) 727–734.