Aptamer-based cell-free detection system to detect target protein

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ABSTRACT

Biomarkers of disease, especially protein, show great potential for diagnosis and prognosis. For detecting a certain protein, a binding assay implementing antibodies is commonly performed. However, antibodies are not thermally stable and may cause false-positive when the sample composition is complicated. In recent years, a functional nucleic acid named aptamer has been used in many biochemical analysis cases, which is commonly selected from random sequence libraries by using the systematic evolution of ligands by exponential enrichment (SELEX) techniques. Compared to antibodies, the aptamer is more thermal stable, easier to be modified, conjugated, and amplified. Herein, an Aptamer-Based Cell-free Detection (ABCD) system was proposed to detect target protein, using epithelial cell adhesion molecule (EpCAM) as an example. We combined the robustness of aptamer in binding specificity with the signal amplification ability of CRISPR-Cas12a’s trans-cleavage activity in the ABCD system. We also demonstrated that the ABCD system could work well to detect target protein in a relatively low limit of detection (50–100 nM), which lay a foundation for the development of portable detection devices. This work highlights the superiority of the ABCD system in detecting target protein with low abundance and offers new enlightenment for future design and development.

1. Introduction

Nature has evolved a vast range of proteins in performing physiological functions to maintain the life activities on earth, and some proteins carrying out a certain function may indicate the proceedings of disease. Such protein could be sorted into biomarkers, which have been regarded as the targets of disease detection and treatment in recent years [1–3], and thus detecting those biomarkers of protein-type remains a long-standing goal in biological and medical fields. To detect a particular protein in a complex sample, determination after separation and binding assays characterizations have been widely used previously [4]. The former approach is a time-consuming method that includes gel filtration chromatography, ion-exchange chromatography, and nickel column purification. However, the latter one also faces a dilemma with low/none binding specificity to the target of interest despite being more convenient. For instance, a typical representative method of enzyme-linked immunosorbent assay (ELISA) is often powerless and frustrating when lacking a specific antibody to the targeted protein. Besides, the low abundance of the target of interest and massive samples also call for a method with high specificity and efficiency.

In terms of binding assays, aptamers own more excellent performance than antibodies as affinity ligands to recognize specific proteins. Aptamers are short, synthetic single-stranded oligonucleotides (DNA or RNA) that can bind to target molecules with high affinity and specificity [5]. These oligonucleotides are commonly selected from random sequence libraries, using the technique called systematic evolution of ligands by exponential enrichment (SELEX) [6]. As a kind of functional nucleic acid, aptamer certainly harbors the nature of nucleic acid which is incomensurable to protein. Particularly, compared with proteins (or antibodies) used in the immunological analysis, nucleic acids own a feature that could be easily amplified via various approaches. Besides, aptamers have a longer shelflife, improved thermal stability, and could
be modified and conjugated more easily, which enable them to become a powerful tool in the biochemical analysis [7].

Here, we took the advantage of aptamer and trans-cleavage activity of CRISPR-Cas12a [8,9] to construct a protein detection system named Aptamer-Based Cell-free Detection (ABCD) system, in which the aptamer was employed as a recognition probe and CRISPR-Cas12a was regarded as a signal amplifier. We demonstrated that this system works well in detecting target protein with considerable sensitivity and specificity, which has the potential to be further used in the biological and medical fields.

2. Materials and methods

2.1. Oligonucleotides used in this study

As shown in Table 1, the crRNA-C3 was synthesized by Sangon Biotech (Shanghai), and the other sequences were synthesized by Borui Biotech (Xiamen).

2.2. Reagents and buffers

The nucleic acid dye Super Gelblue (10,000× ) was bought from Yuheng Biotech (Suzhou). The streptavidin-coated Fe_{2}O_{4} magnetic beads were bought from PuriMag Biotech (Xiamen). The human recombinant epithelial cell adhesion molecule (EpCAM) protein was bought from Sino Biological Inc. And the AsCas12a protein was bought from Huicheng Biotech (Shanghai). DNase I was bought from Beyotime Biotech (Shanghai). The premix powder of 1× PBS was bought from Sangon Biotech (Shanghai). Duplexing Buffer (used in forming double-strand complex) was prepared by adding 5 mM MgCl_{2} into 1× PBS buffer. Washing and Binding Buffer (used in washing magnetic beads and binding double-strand complex onto beads) contained 20 mM Tris-HCl, 1 M NaCl, 1 mM EDTA, 0.02% Triton X-100; pH 7.8. Incubation Buffer (used in binding aptamer and target protein) was prepared by adding 0.05 mM MgCl_{2} into 1× PBS buffer. 5× Trans-cleavage Buffer (used in reactions that Cas12a involved) contained 100 mM Tris-HCl, 500 mM KCl, 5 mM MgCl_{2}, 5 mM DTT, 25% glycerol, 250 μg/mL heparin; pH 7.5. All buffers should be filtered via 0.22-μm filters before being used.

2.3. Molecular hybridization and agarose gel electrophoresis

Equal equivalent (100 pmol) of aptamer (SYL3C-Biotin) and C3-FAM/C4-FAM were mixed then heated to 95 °C for denaturing for 10 min. Then anneal to room temperature slowly. Agarose (3%) gel electrophoresis was performed subsequently with nucleic acid dye Super Gelblue (10,000×) was bought from Yuheng Biotech (Suzhou). The streptavidin-coated Fe_{2}O_{4} magnetic beads were bought from PuriMag Biotech (Xiamen). The human recombinant epithelial cell adhesion molecule (EpCAM) protein was bought from Sino Biological Inc. And the AsCas12a protein was bought from Huicheng Biotech (Shanghai). DNase I was bought from Beyotime Biotech (Shanghai). The premix powder of 1× PBS was bought from Sangon Biotech (Shanghai). Duplexing Buffer (used in forming double-strand complex) was prepared by adding 5 mM MgCl_{2} into 1× PBS buffer. Washing and Binding Buffer (used in washing magnetic beads and binding double-strand complex onto beads) contained 20 mM Tris-HCl, 1 M NaCl, 1 mM EDTA, 0.02% Triton X-100; pH 7.8. Incubation Buffer (used in binding aptamer and target protein) was prepared by adding 0.05 mM MgCl_{2} into 1× PBS buffer. 5× Trans-cleavage Buffer (used in reactions that Cas12a involved) contained 100 mM Tris-HCl, 500 mM KCl, 25 mM MgCl_{2}, 5 mM DTT, 25% glycerol, 250 μg/mL heparin; pH 7.5. All buffers should be filtered via 0.22-μm filters before being used.

2.4. Fluorescence anisotropy

100 pmol aptamer (SYL3C-FAM) was mixed with 55 pmol EpCAM in Incubation Buffer to the volume of 200 μL. Then the reaction system was incubated at 37 °C for 40 min. The fluorescence intensity of parallel/vertical direction was measured by Shimadzu® RF-6000. The polarizers were from a pair of 3D glasses. Attach a polarizer to the exit of the excitation light, and attach another polarizer to the receiving inlet of the emission light to make sure the polarization directions of the two polarizers were vertical/parallel. The excitation wavelength is 495 nm, and the range of emission wavelength is from 500 nm to 600 nm. The control group used Incubation Buffer to replace the EpCAM in this test.

2.5. Competition reaction

The aptamer (SYL3C-Biotin) and C3-FAM were used to form a double-strand complex by molecular hybridization. Magnetic beads with appropriate volume were used and the beads were washed using the Washing and Binding Buffer according to the manual of beads. Then the Incubation Buffer was added for the competition reaction followed by supplementing 20 pmol EpCAM. After incubation at room temperature for 40 min, the supernatant of the reaction system was removed to measure fluorescence intensity by Shimadzu® RF-6000. The excitation wavelength is 495 nm, and the range of emission wavelength is from 500 nm to 600 nm. The Positive Control was C3-FAM to show the obvious high fluorescence signal.

2.6. Trans-cleavage activity of Cas12a

Various concentrations (20, 2, and 0.2 pmol) of C3 were used to activate the trans-cleavage activity of Cas12a. 1 pmol AsCas12a, 2 pmol crRNA-C3, and ssDNA reporter were pre-mixed for 30 min at room temperature. Then C3 and 5× Trans-cleavage Buffer were added to reach a final volume of 200 μL. The fluorescence intensity was measured immediately by Tecan Infinite® M200 Pro. The excitation wavelength is 485 nm, and the emission wavelength is 535 nm. Recording time was set as 440 s and interval time was set as 20 s. DNase I was used as the Positive Control to cut off the ssDNA reporter in this test.

2.7. Integrated experiment

The aptamer (SYL3C-Biotin) and C3 were used to form a double-strand complex by molecular hybridization. Magnetic beads of appropriate volume were used and the beads were washed using the Washing and Binding Buffer according to the manual of beads. Then the Incubation Buffer was added for the competition reaction followed by supplementing varying moles (10, 15, and 20 pmol) of EpCAM. After incubation at room temperature for 40 min, the supernatant of the reaction system was removed to add into the pre-mix of 1 pmol AsCas12a, 2 pmol crRNA-C3, and ssDNA reporter in 5× Trans-cleavage Buffer. The fluorescence intensity was measured immediately by Tecan Infinite® M200 Pro. The excitation wavelength is 485 nm, and the emission wavelength is 535 nm. Recording time was set as 1180 s and interval time was set as 20 s.

2.8. Data analysis and fitting

Statistical data analysis and fitting were performed with the OriginPro 2021 (Learning Edition).

Table 1

| Sequence Name | 5' modification | Sequence (5'→3') | 3' modification |
|---------------|----------------|-----------------|----------------|
| SYL3C-Biotin  |                |                 | TEG-Biotin     |
| SYL3C-FAM    |                |                 | FAM            |
| C3-FAM       |                |                 | FAM            |
| C4-FAM       |                |                 | FAM            |
| C3           |                |                 | FAM            |
| ssDNA Reporter | 6-FAM          |                 | IABKFS         |
| crRNA-C3     |                |                 | IABKFS         |

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3. Results

3.1. Design of ABCD system

Generally, the binding of the aptamer to its target molecule depends on the correct conformation of the aptamer. Therefore, the conformational change during the recognition process becomes a key point to kick off the downstream signal transfer or transformation. This is the basis for designing our ABCD system (Fig. 1). To detect conformational change resulting from the binding of target protein, we utilized a single-stranded DNA with a partial sequence complementary to the aptamer sequence (hereafter called the complementary strand) as a molecule for signal transformation. The aptamer primarily formed a stable double-strand complex with the complementary strand, and then was fixed on a solid phase. The aptamer would bind to the target protein, enter into the detection system and then release the complementary strand. Although the free complementary strand was available to detect, we planned to implement another novel method designed with a signal transformation to make this system possess better performance. Given the trans-cleavage activity that the Cas12a harbors, we determined to trigger this activity of Cas12a by releasing a complementary strand. Once being activated, the Cas12a would cut off the ssDNAs in the system in an unspecific way, including the ssDNA reporter, resulting in the fluorescence recovery of the fluorophore in the ssDNA reporter. This design would achieve the signal transformation from free complementary strand to fluorescence signal, which leads to signal amplification. For the entire workflow, the target protein was signal input while the fluorescence was signal output, undergoing double signal transformations. After trans-cleavage for a certain time, the target protein’s concentration was measured by the fluorescence intensity. Thus, in our experiment, we chose EpCAM as our target protein, which was overexpressed in most solid cancers and represented an ideal tumor antigen candidate on the surface of circulating tumor cells (CTCs) [10], and the identified aptamer SYL-3C [11] to verify the feasibility of our ABCD system.

The binding of the target protein to aptamer triggered conformational change and resulted in the release of the complementary strand. In other words, the complementary strand was out-competed because of the higher combining capacity of the target protein. To make this “competition” reaction available in thermodynamics, it was necessary to investigate the influence of the nucleotide’s number in the complementary strand on the reaction, which was complementary to the aptamer’s sequence (Fig. 2A). Based on SantaLucia’s work [12], we calculated the Gibbs free energy, of which the nucleotides were 9, 10, 11, 12, or 13 in complementary strand, then converted them into disassociated constant \( K_d \) (Fig. 2B) for convenient comparison with that of the aptamer-target protein complex (indicated with the red line). We figured out that when the complementary nucleotides are over 10, the \( K_d \) of the double-strand complex was lower than that of the aptamer-target protein complex, which was unfavorable for the “competition” reaction. However, the fewer complementary nucleotides would lead to an unstable double-strand complex, which was also negative for the “competition” reaction. Thus, molecular hybridization was employed to verify the lower limit numbers of nucleotides in complementary strands steadily complementary to the aptamer sequence. As shown in the results of agarose gel electrophoresis, the bands of double-strand complexes (upper bands) were visibly displayed when the number of complementary nucleotides was more than 9 (Fig. 2C), which indicated that the number of 10 was the lowest limit to not only maintain a stable double-strand complex but also trigger the “competition” reaction.

3.2. Competition: complementary strand is released due to the binding of the target protein to the aptamer

In this research, streptavidin-coated Fe\(_3\)O\(_4\) magnetic beads were employed as the solid phase to fix the double-strand complex, on which the “competition” reaction happened. Since the aptamer sequence SYL-3C was 3′-biotin modified in advance, the binding to streptavidin on the surface of magnetic beads were easy to obtain. Before binding, molecular hybridization was performed to form a double-strand complex. And after binding, we utilized the magnetic field to remove the excess free...
duplexes or single strands to avoid their interferences with subsequent “competition” reactions. In simple tests of the “competition” reaction, complementary strands with 3'-FAM modified were used to detect the fluorescence intensity of supernatant after the reaction for convenience.

We tested the complementary strand C3 with 10 nucleotides complementary to SYL-3C. The results showed that the fluorescence intensity at about 525 nm of supernatant in the “Aptamer + C3 + EpCAM” group was higher than that of “Aptamer + EpCAM” and “Aptamer + C3” groups, and the value of fluorescence intensity of “Aptamer + EpCAM” was similar to that of the blank group (Fig. 3). This indicated that the complementary strand C3 could be out-competed by target protein EpCAM and then released to the supernatant, which was corresponding to the prediction of computational results (Fig. 2B). Though there might be nonspecific adsorption of C3 since the fluorescence intensity of the “Aptamer + C3” group showed a little bit higher than the blank group, the success of “competition” was not concealed. In addition, we have also tested the specificity of the aptamer according to the fluorescence anisotropy (FA) approach [13] (Fig. S1). The ΔFA between the “Aptamer + EpCAM” group and the “Aptamer” group (FA (Aptamer + EpCAM) − FA(Aptamer)) was about 0.113, which indicated that the EpCAM could lead to a significant increase in the FA value of the reaction system. This suggested that the specificity of SYL-3C to EpCAM was high enough, which laid the foundation for subsequent characterizations.

### 3.3. Trans-cleavage activity of Cas12a triggered by complementary strand

The complementary strand C3 was designed complementary to the crRNA with 21-nucleotides, which could trigger the Cas12a-crRNA assembly in theory [8,9]. To test whether the C3 could actually activate Cas12a or not, we used an ssDNA reporter modified with 5'-FAM and 3'-IABkFQ which was commonly used before. The nonspecific cleavage of ssDNA reporter would cause the lapsed quencher and detectable fluorescence. The fluorescence intensity of different incubation times was recorded using a microplate reader to investigate the effect of complementary strand C3 with different concentrations (different moles in the same volume) on fluorescence intensity (Fig. 4). We demonstrated that the fluorescence intensity showed a similar increasing trend with 20 pmol and 2 pmol C3 in 200 μL reaction volume, while no significant increase of fluorescence intensity was observed with 0.2 pmol C3 (DNase I was set as Positive Control). That meant 100 nM and 10 nM C3 could activate the trans-cleavage activity of Cas12a while the concentration of 1 nM C3 could not activate Cas12a in an acute way. In general, the results suggested that the C3 and the crRNA we designed could work well and the Cas12a could be activated to cut off the ssDNA reporter nonspecifically.

### 3.4. Proof of concept: ABCD system works well to detect target protein

After verifying the feasibility of each part in the ABCD system step-by-step, we further performed an integration experiment to detect the target protein EpCAM in accordance with the ABCD system’s operation workflow (Fig. 5A). We used complementary strand C3 as the molecule of first signal transformation, whose performance was validated before. Different from the verification tests in the “competition” reaction, the C3 strand used here was not modified on both ends. After the “competition”
reaction, the supernatant extracted was directly mixed with the pre-assembled Cas12a-crRNA reaction system (containing ssDNA reporter) and then detecting fluorescence intensity immediately. EpCAM with different concentrations was tested, and the fluorescence intensity was plotted against time (Fig. 5B). We found that fluorescence intensity increased almost as a linear mode through time, and a higher concentration of EpCAM had a larger increasing rate of fluorescence intensity. This indicated that the ABCD system could detect target protein EpCAM and distinguish the concentrations of target protein at a relatively lower limit of detection (50–75 nM). These data demonstrated that the ABCD system constructed was able to detect target protein specifically, which presented a good application potential for more complicated biochemical analysis.

4. Discussion

Aptamers is functional nucleic acid that has been widely used in various biochemical analyses. Matthew R. Dunn and colleague analyze 1003 in vitro experiments and revealing new insights into the targets chosen for aptamer selections. Among them, proteins constitute the largest target category with 584 entries, followed by small molecules and cells with 234 and 141 entries, respectively [14]. Protein-targeted aptamer repertoire is much smaller than that of antibodies, while the feature of easy modification, thermodynamic stability, and easy chemosynthesis still attracted flock of researcher’s attention. Among them, the stability of DNA aptamers was much higher than that of RNA due to its chemical property. However, to overcome this dilemma, a wealth of synthetic chemical method has been introduced into the SELEX and post-SELEX modification process to improve the stability of aptamers [15]. For example, 2′-fluoro-, 2′-amino-, and 2′-O-methyl-substituted libraries modification make the aptamers more stable in biological application. Thus, modifications of nucleic acids improve the stability of aptamers, which could be a solution to the low stability of RNA-based aptamers.

The low stability of crRNA not only limited its application, but may became the biggest challenge for practical application of the ABCD system. However, it is worth noting that many methods have been constructed to improve the stability of crRNA. For example, the modification of RNA’s nucleotides with functional groups would increase its stability. Though there is no example for Cas12a now, chemical modification has been proved efficient in increasing Cas9’s gRNA stability [16] and even reducing off-target cleavage without sacrificing on-target activity [17]. Although modified position in the gRNA that directly interface with Cas9 has been shown to reduce its activity, the remainder position of the gRNA sequence tolerates heavy modification [18]. Thus, we have reasons to believe that modified crRNA of CRISPR-Cas12a could also enhance the stability as well.

ELISA method also has the ability to measure EpCAM with relative high sensitivity in the pg/mL level. However, no matter what origins of EpCAM (CTCs/EVs in blood or soluble type in serum) [19–23], the 3–5 h and even overnight coating operations process make it a time-consuming method compared to ABCD system (<2 h). Besides, compared to colorimetric substrate, the more sensitive fluorogenic substrate, such as ampiflu, worked not so well with HRP/H₂O₂ system due to oxidation of the substrate to non-fluorescent resazurin in the presence of hydrogen peroxide [24]. But our ABCD system is not affected by this issue because there is no redox system employed. To be frank, our system should be further improved to decrease the detection limit (such as using the CONAN system [25] or extending the crRNA [26]), however, the time-saving operation and low-cost of our system could not be neglected, while the lower thermodynamics and cross-reactivity of antibodies used in ELISA could not be neglected as well.

The “competition” reaction is the critical part of the ABCD system whose feasibility in thermodynamics should be considered primarily. Because successful “competition” reaction was the necessary prerequisite for valid fluorescence intensity detection. The complementary

**Fig. 4.** Varying moles of C3 was used to activate the trans-cleavage activity of Cas12a. Fluorescence intensity was measured immediately at 535 nm emission wavelength excited at 485 nm after triggering. Data points of every group were fitted via Gauss function to simply show the increasing trend of fluorescence intensity. DNase I was implemented as the Positive Control in this test.

**Fig. 5.** Proof of concept. (A) The operation workflow of the ABCD system employed for detect EpCAM. (B) Varying moles of EpCAM were used to test the performance of the ABCD system. Fluorescence intensity was measured immediately at 535 nm emission wavelength excited at 485 nm after triggering. Recording time was set as 1180 s and interval time was set as 20 s. Linear fitting was performed to show the change of fluorescence intensity through time.
stranded binding to aptamer depends not only on the number of complementary nucleotides but also the salt concentration, which was taken into consideration in our calculation part. Various cases are containing the idea of “competition”, However, we noticed that the feasibility in thermodynamics and the influence from the complementary nucleotides number hadn’t been carefully investigated many times. To clearly present the calculation process and make it convenient for other researchers, we integrated our calculation process and defined an ABKey function explained in a flow chart (Fig. S2) in supplementary. We could find the appropriate range of complementary nucleotides number via calculation following the flow chart when the disassociated constant of the target molecule (not limited to protein)-aptamer complex and the identified sequence of the aptamer is known. It is worth mentioning that we have considered the kinetics of the “competition” reaction, whereas kinetics experiments are difficult for us to implement. We had to use the incubation time in the literature [11] as a reference.

To verified each part in ABCD systems, the “modular” idea acted as guidelines to implement the experiment step-by-step. We divided the ABCD system into the sensor (double-strand complex), the amplifier (Cas12a), and the reporter (ssDNA reporter), which was more in line with the concept of synthetic biology. The input signal of the target protein was transformed through the ABCD system and successfully outputted the fluorescence signal at last.

Recently, researchers have developed a variety of approaches to detect ions [27], small molecules [27,28], nucleic acids [29–33], and transcriptional factors [34] (proteins [33]) based on the assistance of CRISPR-Cas12a. Xiong et al. [27] reported a versatile detection tool combining functional DNA and Cas12a, which harbored the “competition” idea as well. Different from us, besides using aptamer to detect target molecule (ATP), they also utilized another functional nucleic acid DNAzyme to detect Na+. Interestingly, they designed an activation strand to bind two identical aptamer sequences to make the “competition” reaction feasible. Although such a reaction is a homogeneous reaction, we believe that the ABCD system will be more advantageous in eliminating the interference of free nucleic acids. We applied the solid phase in our design of the ABCD system. For convenient operation, the magnetic beads were chosen as the solid phase in our experiments. However, such solid-phase is not limited to magnetic beads. For instance, the microfluidic chip or electrode [33] are fine options to consider, where the double-strand complex can be immobilized by well-developed technology. In addition, diverse compartments could be set to spatially separate the “competition” reaction and the trans-cleavage reaction so that the potential interference of the two reactions would be reduced. For detecting multiple molecules (ions, small molecules, nucleic acids, proteins, cells even vesicles) simultaneously, we could take the work of Ackerman et al. [35] as a reference by applying different types of Cas proteins harboring trans-cleavage activity and orthogonal reporter ssDNAs or ssRNAs, which facilitated multiplexed detection. The premise is to obtain the sequences of aptamers of corresponding target molecules that are to the benefit of tremendous SELEX works. These all present the potential of the ABCD system to be further developed into portable detection devices.

In actuality, we found that the measurement of fluorescence intensity was not so steady when the concentrations of target protein were close to zero (data not shown), which affected the determination of LOD (limit of detection). Similar to other biochemical analysis approaches based on aptamers, ABCD system’s stability may be affected by salt concentration, pH, and temperature. Using the same buffer conditions and temperature in aptamer selecting will greatly ameliorate the problems. Additionally, identifying new aptamers with higher affinity and specificity to the target molecules is able to improve the ABCD system’s efficiency. We will endeavor to build up the quantitative analysis performance of the ABCD system in the next stage and demonstrate the feasibility in the real samples (such as plasma) in the future.

5. Conclusions

In conclusion, we constructed an Aptamer-Based Cell-free Detection System, in which the robustness of aptamer in binding specificity and the signal amplification ability of CRISPR-Cas12a’s trans-cleavage activity was combined to detect the target protein (EpCAM). More importantly, the ABCD system could work well to detect target protein with a relatively low limit of detection (50–100 nM), which could be further developed for portable detection devices. The Aptamer-Based Cell-free Detection System development in this work offers new enlightenment for the future design and development of cell-free detection process with higher accuracy and specificity.

Author contributions

B.F. and A.H.Z. designed the experiments and co-supervised the project. J.H.C. and A.H.Z. wrote the manuscript with inputs from all authors. J.H.C., X.Y.Z., J.Y.Z., and R.F.Y carried out the experiments and analyzed the data. F.W. performed the theoretical calculation. All authors conceived the work and discussed the experiments.

CRediT authorship contribution statement

Junhong Chen: Methodology, Writing – original draft. Xiaoyan Zhuang: Investigation, Validation. Jiyang Zheng: Visualization. Ruofan Yang: Methodology, Fei Wu: Software. Aihui Zhang: Project administration, Writing – review & editing. Baishan Fang: Conceptualization, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

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