Sensitive and Specific Neutrophil Gelatinase-associated Lipocalin Detection by Solid-phase Proximity Ligation Assay

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Neutrophil gelatinase-associated lipocalin (NGAL) is a candidate diagnostic biomarker for acute kidney injury (AKI). Since there is no specific treatment to reverse AKI, a good biomarker such as NGAL can increase the performance of clinical care. Therefore, a timely, specific and sensitive assay for detecting NGAL is critical for clinical determination. In this study, we established a solid-phase proximity ligation assay for the detection of NGAL using polyclonal antibodies conjugated with a pair of oligonucleotides. The data are read out as the Ct value via quantitative real-time polymerase chain reaction (qPCR). Our results demonstrate that this new assay performs with good specificity and sensitivity for detection of NGAL spiked in buffer or serum, which indicates that the solid-phase proximity ligation technique is a promising tool for diagnostics in clinical decisions.

Keywords Neutrophil gelatinase-associated lipocalin, solid-phase proximity ligation assay (SP-PLA), diagnostic biomarker

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Introduction

Acute kidney injury (AKI) was called acute renal failure (ARF) previously,1 which meant an abrupt loss of kidney function that develops within seven days.2 The traditional biomarker for AKI is the serum creatinine, but it is quite limited in its sensitivity and it can be influenced by various extra renal factors.3,4 Studies have shown that neutrophil gelatinase-associated lipocalin (NGAL) plays an unambiguous role in the early diagnosis of AKI.4 NGAL is a ubiquitous protein of 178 amino acids. NGAL belongs to the lipocalin family, in which the proteins transport steroids and lipids.5 NGAL can be measured in whole blood, serum, plasma, and urine.6 Serum creatinine could be upregulated in a few days after the injury, while NGAL could indicate AKI in only several hours after an insult, which may significantly improve the outcome and reduce mortality of AKI.4 Thus, to find a sensitive and specific assay for detecting NGAL is critical for clinical determination.

Proximity ligation assay (PLA) is a DNA-mediated affinity-based assay that uses oligonucleotides attached to affinity reagents, as reporter molecules.7–9 PLA can be used to sensitively and specifically detect target proteins. We applied solid-phase PLA to detect NGAL. The target protein (NGAL) is captured via an antibody immobilized on the solid support such as magnetic microparticles. These affinity antibodies are coupled to oligonucleotides. When the NGAL protein is recognized by a pair of such antibodies, the attached DNA can be brought into proximity.

Experimental

Plasmids, bacterial strains, growth and induction conditions

The synthetic NGAL gene (Generay Biotechnology, Shanghai, China) was 549 bp in the pET32a vector, and then was transformed into E. coli BL21 to express protein. The bacteria were grown in 5 mL Luria-Bertani (LB, Sangon Biotech, Shanghai, China) Broth medium supplemented with 100 mg mL⁻¹ ampicillin at 37°C and shaking at 220 rpm overnight.

NGAL protein expression and purification

To express soluble NGAL recombinant protein, large-scale production was performed in shaker flasks with growing bacteria in LB medium supplemented with 100 mg mL⁻¹ ampicillin until OD600 reached between 0.6 and 0.9. NGAL recombinant
protein expression was induced with 1 mM IPTG for 4 h at 30°C. After inducing, the cells were centrifuged with 8000 rpm for 15 min at 4°C. The lysis of cells was prepared using a high-pressure homogenizer. And then the lysate solution was centrifuged at 8000 rpm for 15 min at 4°C. The supernatant was collected for purification.

The supernatant was binding with 2 mL Ni-NTA beads at 4°C for 1 h and purified by a ninitrilotriacetic acid (Ni-NTA) superfow Sepharose column. The proteins/beads were washed with buffer containing lower concentrations of imidazole (0, 20, and 50 mM), and eluted with different concentrations of imidazole (100, 250, and 500 mM). The eluted proteins were desalinated on a Millipore column (Millipore, Bedford, MA) with a molecular mass cut-off of 3 kD and dialyzed in PBS. Protein purity was detected by Coomassie-stained sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and the concentration was determined by Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Immunization and generation of polyclonal antibody against NGAL

The polyclonal antibody in this experiment was produced by a professional biotech company (Huibiao Biotech, Nanjing, China). A sheep of at least six months of age was maintained for one week for adaptation. The sheep was immunized with an equal volume of emulsified complete Freund’s adjuvant (FA Sigma-Aldrich, St. Louis, MO) and administered via a primary subcutaneous injection. Booster injections were conducted five times at one week intervals using the same amount of the protein mixed with incomplete Freund’s adjuvant at 1:1 volume ratio. To immunize, 2.5 mg purified NGAL recombinant protein was used each time. After six rounds of immunization, blood was harvested by heart puncture. And then the blood was incubated at 4°C for 1 h, and centrifuged with 8000g for 30 min at 4°C. Serum was stored at –80°C. Finally, the polyclonal antibody was purified from 1000 mL serum samples by affinity chromatography using NGAL-conjugated affinity columns (NHS-activated Sepharose 4 Fast Flow, GE Healthcare Life Sciences).

Preparation of proximity probes and antibody

The proximity probes were synthesized by Integrated DNA Technologies (IDT). Arm1 oligonucleotide sequence is 5′-azide-

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\text{CGC ATC GCC CTT GGA CTA CGA CTG ACG AAC CGC TTT GCC TGA CTG ATC GCT AAA TCG TG} \text{-} 3′
\]

Arm2 oligonucleotide sequence is 5′-azide-TCG TGT CTA AAG TCC GTT ACC TTG ATT CCC CTA ACC CTC TTG AAA AAT TCG GCA TCG GTG A-3′. Then, 10 μL of polyclonal antibody against NGAL (2 μg μL⁻¹) was incubated with 1 μL of 4 mM dibenzylcyclooctyne-NHS ester (DBCO, Jena Bioscience, Jena, Germany) dissolved in DMSO for 30 min at room temperature. To this it was added 1 μL of 1 M Tris-HCl pH 8.0 for 5 min at RT to stop the reaction. The free DBCO was removed by a Zeba spin desalting column (Thermo Fisher Scientific, Waltham, MA). The antibodies were divided into two equal parts. The antibodies were respectively mixed with 100 μM Azide-modified oligonucleotides Arm1 and Arm2 and incubated overnight at 4°C under rotation. The antibody-oligonucleotides were diluted to 500 nM (antibody) concentration in PBS with 0.1% BSA and 0.02% NaN₃.

Solid phase PLA detection

The antibodies were coupled to Dynabeads® M-270 Epoxy (Invitrogen Life Technologies, Carlsbad, CA) overnight at 37°C while rotating. The assay was performed according to the protocol of the manufacturer. Dilution series of antigens were prepared in PLA buffer of 1 mM D-biotin (Invitrogen Life Technologies), 0.1% BSA (New England Biolabs), 0.05% Tween20 (Sigma-Aldrich), 100 nM goat IgG (Sigma-Aldrich), 0.1 μg μL⁻¹ salmon sperm DNA (Invitrogen Life Technologies),
5 mM EDTA in PBS or 10% Fetal Calf Serum (FCS). We added the different concentrations of antigens, including a negative control where there was no protein added, to the antibody-beads capturers and incubated for 1.5 h at RT. The microspheres were collected on a 96-well plate magnet and washed twice with PBST (PBS with 0.25% Tween 20). Next, 25 nM of the antibody-oligonucleotides conjugates were added into each well and incubated for 1.5 h at RT and washed twice. For each 50 μL solid-phase PLA reaction, we used 1xPCR buffer (Quanta, Gaithersburg, MD), 2.5 mM MgCl₂, 0.2 mM dNTP (U), 0.08 mM forward primer (the sequence is 5'-CAT CGC CCT TGG ACT ACG A-3'), 0.1 μM reverse primer (the sequence is 5'-GGG AAT CAA GGT AAC GGA CTT TAG-3'), 0.1 μM connector oligonucleotides (the sequence is 5'-TAC TTA GAC ACG ACA CGA TTT AGT TT-3'), 0.22 μM TaqMan probe (the sequence is 5'-TGA CGA ACC GCT TTG CCT GA-3'), 0.01 U μL⁻¹ Taq polymerase (Quanta), 0.01 U μL⁻¹ T4 ligase (Thermo Fisher Scientific), and 0.002 U μL⁻¹ Uracil-N-Glycosylase (UNG, Thermo Fisher Scientific).

The reactions included an initial incubation for 5 min at 95°C, and then 15 s at 95°C and 1 min at 60°C, repeated 45 times. All qPCRs were performed on ABI PRISM 7700.

**Results and Discussion**

**Expression and purification of NGAL protein**

We expressed the NGAL protein by using *E. coli* BL21 cells transformed with confirmed plasmid construct described above. The NGAL recombinant protein, which has the His-Tag, was purified with Ni-nitrilotriacetic acid (Ni-NTA) Sepharose column and determined by SDS-PAGE followed by Coomassie brilliant blue staining. In Fig. 2A, the different concentrations of imidazole were used to elute the binding proteins from the Ni-NTA. The yield of purified recombinant NGAL could reach 100 - 150 mg/L of bacterial culture. After further purification (Gel filtration, Äktá, GE Healthcare Life Sciences), the purity of NGAL was significantly improved, and it could be used for the following experiments (Fig. 2B).

**Production and purification of polyclonal anti-NGAL**

After immunization and preparation of the polyclonal against NGAL recombinant protein, the double diffusion dilution method was used to evaluate the level of NGAL in the specific immune response. The result revealed an increase in reactivity of NGAL recombinant protein in the first week and at the end of immunization the serum showed high reactivity, which was able to reach 1:16 - 1:32 by the double diffusion dilution method. The polyclonal antibodies were determined by SDS-PAGE with Coomassie brilliant blue staining (Fig. 3).

**Preparation of PLA probes**

The PLA probes were prepared as described. Since the remaining unconjugated oligonucleotides could be removed by washing in the following step of the solid phase PLA reactions, there was no specific purification for the conjugated probes. The conjugated probes were determined by SDS polyacrylamide gel, followed by protein silver staining (Fig. 4), a shift in molecular weight when compared to the antibodies alone could be observed by the conjugated probes with the NGAL polyclonal antibodies.

**Detection of NGAL by solid-phase PLA**

As described, we developed a solid-phase PLA by using polyclonal antibody for sensitive and specific detection of NGAL. Compared to the standard ELISA, the SP-PLA provides a broader range and a lower limit of detection in the previous
studies. Using the micro particle based SP-PLA, NGAL was first captured by polyclonal antibodies immobilized on magnetic particles and then NGAL was detected by a pair of PLA probes, both utilizing the polyclonal antibody. Figure 5A shows the detection of NGAL spiked in buffer which may simulate NGAL in urine. The result also showed that the dynamic range of this assay extended more than six orders of magnitude, and the detection limit was about 0.064 ng mL\(^{-1}\) (Fig. 5A) using the following formula: \( CT_{LOD} = C_{SN} - 2 \times S_N \). \( C_{SN} \) is the average CT obtained for the background noise and \( S_N \) is the standard deviation of this value. Then we detected the NGAL spiked in 10% serum, which simulates NGAL in blood samples. Figure 5B shows that spiking in serum would not affect the detection of NGAL by our PLA assay, which indicates a clinical application for detection of NGAL in blood samples.

**Discussion**

NGAL is one of the new generation biomarkers for acute kidney injury, which can be measured in whole blood, serum, plasma, and urine by several commercially available analytical immunoassays. However, clinical quality specifications (sensitivity, specificity, positive and negative predictive values, etc.) should be addressed as well as the balance between costs and benefits. Generating a more specific and sensitive method for detecting NGAL would be helpful for improving the clinical outcome and quality of care.

The PLA is a suitable method for NGAL detection. This assay requires simultaneous binding of two or more epitopes of a protein in order to yield detectable signals. In this study, we have raised a polyclonal antibody that subsequently was used in a solid-phase PLA for specific detection of NGAL via recognition by three probes binding different epitopes, one of which was used as the capture probe while the other two were used as PLA probes. The specificity increased because of the requirement to have three probes produce detectable signals, and the DNA-based signal amplification by real-time PCR enhanced the detection signals and hence the assay sensitivity. The combination of high specificity and increased sensitivity has an advantage over the current commercially available assays based on conventional ELISA. The LOD (limit of detection) of a commercial NGAL detection kit (Ab-Match Assembly Human NGAL Kit (MBL C., L.)) is 0.073 ng mL\(^{-1}\), while our SP-PLA assay had a limitation at 0.064 ng mL\(^{-1}\). To estimate the background signal increment and the difference of mean CT value in 10% serum, there are several possible reasons: 1) there may be a trace of NGAL protein in the serum; 2) nonspecific antibody-oligonucleotides conjugates adsorption give rise to background; 3) complex environment of serum can also lead to some unstable factors. The conditions in this assay need to be optimized in the future to reduce the LOD and background.

**Conclusions**

We have raised polyclonal antibodies against NGAL that were used to establish a solid-phase PLA for enhanced detection of NGAL, offering high specificity, sensitivity and a wide dynamic range either spiking in buffer or 10% serum to simulate the different physiological environments. This new assay can be a promising diagnostic tool for infectious diseases.

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