Review

Molecular PINCERs® for biomarker analysis and their potential application in hepatitis C diagnosis

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Molecular PINCERs® are antibody-based protein sensors that allow rapid and sensitive detection of specific proteins in solution. The sensors allow quick detection of a target, with sensitivity and specificity approaching that of conventional immunoassays, but require no sample manipulation other than mixing of the test sample with sensors. Combining the PINCER assay with immunoprecipitation allows quick and sensitive detection of anti-HCV immunoglobulin (Ig)G and IgM in human serum. This improvement is achieved through binding of anti-HCV IgG and IgM from serum to HCV-antigen-immobilized microspheres, followed by quantification of the IgG and IgM using molecular PINCERs. The PINCER-based assay is cost-effective, sensitive and has potential for clinical diagnostic use.

Biomarkers

The term biomarker was introduced in 1989 and was defined in 2001 by the NIH Biomarkers Definitions Working Group as ‘a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic response(s) to a therapeutic intervention’ [1]. Biomarkers can be proteins, nucleic acids, lipids, small metabolites, as well as whole cells. Biomarkers may reveal a variety of health or disease characteristics, and be used for risk assessment, disease prognosis and prediction of treatment response. There are hundreds of mature protein biomarkers that have been identified and characterized. For example, cardiac troponin I (cTnI) and T (cTnT) have been widely used as biomarkers for myocardial damage [2–4]. D-dimer, C-reactive protein (CRP), creatine kinase-MB, and myoglobin are also well-characterized cardiac biomarkers [2,5–8]. Prostate-specific antigen, CA-125 and HER-2/neu tests are clinically used for the screening of prostate cancer, ovarian cancer and breast cancer, respectively [9–11]. Hepatitis B surface antigen and anti-HCV antibodies are widely used serological biomarkers for infection [12,13]. Detection of biomarkers can be accomplished by a wide variety of methods, including biochemical analysis, serological and virological methods, proteomics approaches, as well as biomedical imaging. To measure protein biomarkers in a biological fluid, the enzyme immunoassay and enzyme-linked immunosorbert assay (ELISA) have been widely used. Low-abundance proteins are usually detected with a sandwich ELISA, in which the antigens are captured by capture antibodies immobilized on the solid surface, and probed with detection antibodies. With more sensitive chemiluminescence substrates or amplification approaches, such as tyramide signal amplification and rolling circle DNA amplification [14–17], pg/ml-level sensitivity can be reached in these assays. Despite the high sensitivity, a format such as ELISA is a heterogeneous assay requiring a number of manipulations and up to several hours to perform. Reducing the complexity as well as the cost of assay procedures is critical to immunoassay development, especially for point-of-care diagnosis.

Molecular PINCERs® in biomarker analysis

Molecular PINCERs® are homogenous antibody-based protein sensors that allow rapid and sensitive detection of a specific protein in solution [18]. Figure 1 illustrates the general design of the homogenous PINCER assay. A pair of antibodies recognizing non-overlapping epitopes of the target protein are functionalized by attaching short complementary oligonucleotides via nanometre-scale flexible linkers. Each oligonucleotide is labelled with a donor or acceptor fluorochrome that participates in fluorescence-resonance-energy-transfer
(FRET) [19]. In the presence of the target analyte, both antibodies will bind to the target molecule, resulting in a large increase of the local concentration of signalling oligonucleotides. This, in turn, will drive the annealing of the oligonucleotides and bring the fluorophores to close proximity, resulting in efficient FRET and producing a fluorescence signal that is proportional to the amount of target analyte present in the samples.

Based on this assay platform, several biomarker-specific PINCER assays have been developed with desirable sensitivity, specificity and accuracy, including one for CRP whose concentration of CRP in serum is normally about 0.8 μg/ml but following an acute-phase stimulus may reach up to 500 μg/ml [20]. The level of cTnI in the blood of a healthy adult is almost negligible, but after acute myocardial infarction, it can reach a peak level of 100 ng/ml within 18 h [21]. Using the PINCER assay, CRP and cTnI have been measured in serum and plasma to sensitivity of 2 ng/ml (17 pM) and 1 ng/ml (40 pM) achieved, respectively [18], levels that are far below their levels under disease conditions. PINCER assays were also developed to detect human C-peptide and insulin, with a lowest limit of detection (LOD) of 50 pM for C-peptide and 100 pM for insulin. The LOD is sufficient to detect C-peptide in serum (in which the normal range is 1.1–3.2 ng/ml [22]) and insulin in the pancreas (whose average concentration is about 10 μM [23]). PINCER assays are also useful tools for the detection of insulin and C-peptide secreted from cultured islets [24]. Human serum albumin (HSA) is regarded as a non-specific biomarker for several diseases, such as nephrosis, liver disease, gastrointestinal protein loss, shock, oedema and diabetes. The normal concentration of HSA in human serum, urine and saliva is 35–50 mg/ml, 2.2–25 μg/ml and <0.5 mg/ml, respectively [25]. Changes in HSA levels reflect disease conditions. The sensitivity of the PINCER assay for HSA can reach 3.9 ng/ml, which is sufficient for quantitative analysis of HSA in the various biological samples [26]. A systematic analysis of HSA levels using the PINCER assay was performed with randomly chosen saliva, urine and serum samples, and the results were validated by ELISA. The HSA PINCER assay exhibited excellent accuracy and robustness for all three types of biological

**Figure 1.** Overall design of the PINCER<sup>®</sup> assay

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**FRET**, fluorescence-resonance-energy-transfer.
samples, with intra- and inter-assay coefficient of variation (CVs) <10% and majority <5% [26]. The standard curves of the PINCER assay for human CRP, insulin, C-peptide and albumin are shown in Figure 2. For all these PINCER assays, minimal sample manipulation is required. Maximal fluorescence signal is usually reached within 20 min after mixing analytes with assay solution, and the signal remains stable for >2 h.

Multiplexing also may be achieved with the homogeneous PINCER assay design. This requires the PINCERs for each analyte to be constructed using different pairs of fluorescence probes which have fluorescence excitation, emission or both, at different wavelengths. For example, by mixing FAM/Oyster645-labelled CRP PINCER pairs and Eu3+/Alexa647-labelled human immunoglobulin (Ig)G PINCER pairs in a single reaction, we were able to simultaneously detect CRP and IgG at 50 ng/ml level, with an average recovery rate of 98.8% (Additional file 1).

**Molecular PINCER® in HCV diagnosis**

Early diagnosis of HCV infection is crucial for timely treatment and curtailment of its spread. Over 170 million individuals are infected with HCV globally, with 3–4 million new infections occurring each year. In the United States, it is estimated that 1 in 33 baby boomers have the disease, and mortality rates associated with HCV are currently rising. HCV has been described as a ‘silent epidemic’; though the disease is treatable and manageable in the acute stage, it may be asymptomatic in up to 85% of cases. When allowed to progress

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Figure 2. Quick detection of biomarkers using PINCER® assays

![Graphs A-D](image-url)
unchecked, HCV infection has become a leading cause of end-stage liver disease and liver cancer. Affordable and accessible screening for acute and active HCV infection would be critical to prevent progression of disease and its spread.

PCR screening and serological assays to detect viral RNA and HCV antibodies in patient blood samples are currently the major approaches to hepatitis C diagnosis [27]. FDA-approved anti-HCV screening kits are mostly immunoassays detecting anti-HCV IgG captured on HCV antigens immobilized on the solid phase. The signals are developed through catalysing colorimetric or chemiluminescent substrates linked to second-layer antibody. Anti-HCV IgG can be detected in 80% of patients within 15 weeks after exposure, and in greater than or equal to 97% by 6 months after exposure [28,29]. A transient IgM antibody response at the onset of hepatitis may be observed in some cases, with the level decreasing after several months [30–32]. Using the proprietary PINCER technology, we have developed homogeneous PINCER assays for human and mouse IgG and IgM, respectively. These PINCER assays exhibit excellent sensitivity and reproducibility, with the lowest detection limits of human or mouse IgG both at 3 ng/ml and CV values <3.5%. The lowest detection limits of human or mouse IgM are both at 15 ng/ml, with CV values <3%. Combining the PINCER assay with immunoprecipitation allows us to develop a quick and sensitive approach to detect both anti-HCV NS4 antigen IgG and IgM in human serum by separating anti-HCV IgG and IgM from serum using HCV antigen immobilized microspheres, followed by quantification of the IgG and IgM using the molecular PINCERs.

For proof of concept, we tested mouse anti-NS4 IgG or IgM spiked into normal human serum. By mixing 3×10⁷ to 1×10⁸ NS4-coated beads with 20 μl diluted serum samples spiked with mouse anti-NS4 IgG or IgM, followed by incubation at room temperature for 1 h and washing away the unbound serum proteins, a spike of 10 nM mouse anti-NS4 IgG or 5 nM mouse anti-NS4 IgM in serum can be detected.

The performance of the assay was further evaluated on a commercially available anti-HCV seroconversion panel. A remarkable increase in the anti-NS4 IgG response at the later stage of infection was observed in the human IgG PINCER assay (Figure 3A). A slight increase in anti-NS4-IgM response was also observed with the progress of the disease in the human IgM PINCER assay, but a clear trend was lacking (Figure 3B). A modified ELISA was performed to validate the PINCER assay results. The human IgG PINCER assay using 5 μl of beads in each reaction showed good correlation (R²=0.988) with results obtained with ELISA (Figure 3C). However, the human IgM PINCER assay showed lower responses compared to the results in ELISA (data not shown). The signal-to-background ratio and sensitivity of the assay can be further improved by constructing molecular PINCERS with higher affinity anti-human IgM antibody, or using brighter fluorescence probe pairs. The recombinant NS4 antigen used for IgM capture contains immunodominant regions of the NS4 protein (amino acids 1691 to 1710, 1712 to 1733 and 1921 to 1940) derived from HCV genotype 1, 2, 3 and 5 strains [33]. IgM antibody, raised against antigen from the capsid, may serve as an alternative acute-phase marker in HCV infection [30]. Using recombinant antigen containing more immunogenetic epitopes or multiple HCV antigens should improve recovery of the anti-HCV antibodies and sensitivity of the assay. Validation employing a larger collection of clinical samples should provide more information on the performance of molecular PINCERS applied to different sample sets varying according to HCV genotypes, degree of viraeemia and severity of hepatitis.

Towards development of homogenous PINCER® assays

The molecular PINCER platform can be adapted to minimize further sample manipulation and assay procedures, which would be highly desirable in diagnostic applications. The approach outlined above already requires one less step of sample handling than is necessary in traditional ELISA assays. This may provide a better antibody recovery rate and assay reproducibility. An even more ideal diagnostic method would be a homogeneous platform that would specifically detect anti-HCV antibodies in serum without being affected by interference of non-specific IgGs and other serum proteins. This platform may be achieved through probing the target antibody using a pair of molecular PINCERS that are constructed from HCV antigens or peptides containing HCV epitopes. Simultaneous binding of the two PINCER pieces to the variable regions on the specific anti-HCV antibody allows PINCER-target complex formation, and thus the production of target-concentration-dependent FRET signal. Other permutations of the molecular PINCER platform could include combining one PINCER constructed from an HCV antigen or epitope-containing peptide, and another constructed from an anti-human IgG (or IgM) antibody. The specific anti-HCV IgG (or IgM) binds to HCV antigens or peptides through its variable region and interacts with anti-IgG (or IgM) antibody through its Fc region. The simultaneous binding by the two PINCERS would allow anti-HCV antibody to be specifically detected. Regardless of design, the assay will involve just mixing the PINCERS with serum samples, with the FRET signal being read after incubation at room temperature for 30 min. Given that there are at least six
major genotypes of HCV, and that antibodies are developed in patients against structure and non-structure proteins, using a single antigen may miss detection of certain antibodies and give false-negative results. The molecular PINCERs could use a population of HCV antigens or multi-epitope representing all 6 genotypes, so improving sensitivity.

Conclusions

The PINCER assays achieve sensitivity levels comparable to immunoassays, but eliminate the variability of detection outcomes in respect of variability imposed by coating of analyte-capture reagents and rinse steps, and are more cost-effective and readily adaptable to both low- and high-throughput screening formats. As long as there are high-affinity reagents available, developed for capturing the target analyte, they may be utilized for PINCER-based diagnostics, including those for hepatitis C.

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Disclosure statement

Y-HC owns major shares of Mediomics. LT is a coinventor of the PINCER technology. REW declares no competing interests.

Additional file

Additional file 1: Supplementary data can be found at http://www.intmedpress.com/uploads/documents/AVT-12-SP-2495_Tian_Add_file1.pdf

References


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