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Real-time immuno-polymerase chain reaction in a 384-well format: Detection of vascular endothelial growth factor and epidermal growth factor-like domain 7

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ABSTRACT

Immuno-polymerase chain reaction (immuno-PCR) combines the specificity of antibodies with the amplification power of PCR to detect low levels of proteins. Here, we describe the development of a 384-well immuno-PCR method that uses streptavidin coated on a PCR plate to capture complexes of biotinylated capture antibody, antigen, and DNA-labeled detection antibody. Unbound molecules are removed by a wash step using a standard plate washer. Antibody–DNA molecules in bound complexes are then detected directly on the plate using real-time PCR. Circulating human vascular endothelial growth factor concentrations measured by this method correlated with measurements obtained from enzyme-linked immunosorbent assay (ELISA). Using this method, we developed an assay for human epidermal growth factor-like domain 7 (EGFL7), an extracellular matrix-bound angiogenic factor. EGFL7 is expressed at a higher level in certain cancers, although endogenous EGFL7 concentrations have not been reported. Our 384-well EGFL7 immuno-PCR assay can detect 0.51 pM EGFL7 in plasma, approximately 16-fold more sensitive than the ELISA, utilizing the same antibodies. This assay detected EGFL7 in lysates of non-small-cell lung cancer and hepatocellular carcinoma cell lines and also hepatocellular carcinoma, breast cancer, and ovarian cancer tissues. This 384-well immuno-PCR method can be used to develop high-throughput biomarker assays.

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Since immuno-polymerase chain reaction (immuno-PCR)[1] was first described two decades ago [1], many different formats have been used to measure very low levels of proteins [2,3]. However, improvements in the throughput and standardization were needed before this technology could be widely used for biomarker assays. Previously, we described a real-time immuno-PCR method utilizing streptavidin-coated PCR tubes in a 96-well format to detect vascular endothelial growth factor (VEGF) [4]. Here we report development of a simple 384-well immuno-PCR method utilizing streptavidin coated on standard real-time PCR plates to capture the complexes of biotinylated capture antibody, antigen, and DNA-labeled detection antibody as uncoated PCR plates do not adsorb proteins well. Use of streptavidin-coated PCR plates enabled the complex capture and the real-time PCR steps to be carried out in a single plate. These plates can be washed using a standard ELISA plate washer, greatly simplifying the wash steps after the capture step. The antibody–DNA molecules in the streptavidin captured complexes are detected using a standard real-time PCR instrument.

EGFL7 is an endothelial cell derived protein that regulates vascular tube formation. Its expression is downregulated in mature vasculature and upregulated in proliferating endothelium [5–7]. Higher EGFL7 expression is correlated with a higher tumor grade and poorer prognosis in certain cancers, including hepatocellular carcinoma (HCC) [8,9]. Overexpressed EGFL7 was found both intracellularly and tightly associated with the extracellular matrix, with very little in the conditioned media [6]. However, endogenous EGFL7 concentrations in biological samples have not been reported. Utilizing our 384-well immuno-PCR method and the antibody pair used in our EGFL7 ELISA, we developed a sensitive EGFL7 assay. Our assay detected EGFL7 in lysates of non-small-cell

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[1] *Abbreviations used:* C_t, cycle threshold; CV, coefficient of variation; EGFL7, epidermal growth factor-like domain 7; ELISA, enzyme-linked immunosorbent assay; HCC, hepatocellular carcinoma; immuno-PCR, immuno-polymerase chain reaction; NSCLC, non-small-cell lung cancer; PBS, phosphate-buffered saline; VEGF, vascular endothelial growth factor.

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lung cancer (NSCLC) and HCC cell lines. We also assayed cancer tissue lysates from patients and detected EGFL7 in HCC, breast cancer, and ovarian cancer tissues. This 384-well immuno-PCR method may be used to quickly convert ELISAs of other biomarkers to more sensitive immuno-PCR assays with little optimization once a detection antibody–DNA is prepared.

Materials and methods

Antibody–DNA conjugation

A 71-mer DNA 5′-NH2-TTCCAGTCCAGGAGAACCAACATAAA CGCCGCAGATACATCCAACGAAAGAGTTGGTGGACAGGAGGTTGG-3′ (Integrated DNA Technologies, Coralville, IA) was activated with *N*-succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Pierce, Rockford, IL, USA). Mouse monoclonal anti-VEGF A4.6.1 IgG_1 or anti-EGFL7 18F7 IgG_{2b} (Genentech) was activated with *N*-succinimidyl-*S*-acetylthioacetate (Pierce, Rockford, IL, USA) and conjugated to the activated DNA at a 1:3 molar ratio following the manufacturer's instructions. Free DNA was removed using a Superose 12 gel filtration column. Free antibody was removed using a Vivapure Q mini column (Sartorius Stedim Biotech GmbH, Goettingen, Germany). The labeling efficiencies for anti-VEGF A4.6.1 and anti-EGFL7 18F7 were 0.95 and 2.5 DNA/antibody, respectively, using BCA protein assay (Pierce) to measure the antibody concentration and PCR to measure the DNA concentration.

Plasma, serum, cell lysate, and tissue lysate samples

Normal K_2EDTA plasma pooled from 25 males and 25 females and normal individual K_2EDTA plasma samples were purchased from Bioreclamation (Westbury, NY, USA). Colon cancer serum samples were purchased from Binding Site (San Diego, CA, USA). NSCLC K_2EDTA plasma samples were collected from patients who participated in a clinical study of anti-EGFL7 parsatuzumab (Genentech). Patient consent was obtained prior to sample collection. Human embryonic kidney 293 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). They were transiently transfected with plasmid expressing full-length EGFL7 cDNA (OriGene, Rockville, MD) using Lipofectamine (Invitrogen, Carlsbad, CA, USA). NCI-H2126, NCI-H1770, HepG2, PLC/PRF/5, and Snu-182 cell lines were purchased from ATCC. HLE, HLF, huH-1, and JHH-2 cell lines were purchased from Japanese Collection of Research Bioresources (Saito, Japan). RERF-LC-MS cell line was purchased from Japan Health Sciences Foundation (Tokyo, Japan). CAL-12T cell line was purchased from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Human cancer tissues were purchased from ConversantBio (Huntsville, AL) or Avaden BioSciences (Scarsdale, NY). Cells and ground tissues were lysed with IP buffer (Pierce) containing protease inhibitors (Roche Applied Science, Indianapolis, IN, USA). Protein concentrations were determined by BCA assay. Total RNA samples were prepared using RNEasy plus kit (Qiagen, Valencia, CA, USA). EGFL7 RNA was measured using Taqman gene expression assay (Applied Biosystems, Carlsbad, CA, USA) in quadruplicate. Relative EGFL7 RNA levels vs the HepG2 EGFL7 RNA level were calculated using the $2^{-\Delta Ct}$ method where $\Delta C_t = (C_t - C_t$ of HepG2 EGFL7 RNA).

A 384-well immuno-PCR method to detect VEGF and EGFL7

Biotinylated capture antibody (0.2 μg/ml), standards or samples, and DNA-labeled detection antibody (0.025 μg/ml) were preincubated for 2 h in sample buffer. The sample buffer consisted of 0.5% bovine serum albumin (BSA), 0.05% polysorbate 20, 15 ppm ProClin, 0.25% CHAPS, 5 mM EDTA, 0.35 N NaCl, 100 μg/ml mouse IgG (Equitech Bio, Kerrville, TX, USA), and 250 μg/ml calf thymus DNA in PBS, pH 7.4. Calf thymus DNA purchased from Sigma (St. Louis, MO, USA) or Invitrogen (Grand Island, NY, USA) was heated to 95 °C for 10 min and then kept on ice. The streptavidin-coated 384-well real-time PCR plate (0.1 ml/well, Roche Applied Science-Custom Biotech, Penzberg, Germany) was blocked for 2 h with 50 μl SuperBlock blocking buffer (Pierce) containing 250 μg/ml calf thymus DNA and 1 mg/ml normal mouse serum protein (Rockland Immunochemicals, Boyertown, PA, USA) in each well. The plate was then mounted on a plate positioner (Bio-Rad Laboratories, Benicia, CA, USA) and washed 3 times with 40 μl/well wash buffer (0.5% BSA, 0.05% polysorbate 20, 15 ppm Proclin in PBS, pH 7.4) on a 384-well plate washer (BioTek, Winooski, VT, USA). An aliquot (20 μl/well) of the antibody–antigen mixture was added to the plate. After 20 min incubation, the plate was washed 3 times with 25 μl/well wash buffer to clean the sample incubation area first, and then 10 more times with 40 μl/well wash buffer. PCR mixture containing 0.9 μM of each primer (5′-CCAGTCCAGGAGA ACCAACATAA-3′ and 5′-TCCTGTCCACCAACT CTTTCG-3′) and 0.25 μM of probe (5′-CCGCAGATACATCCp-3′, labeled with a fluorescent dye FAM and a quencher) in Universal Taqman Master Mix II (Applied Biosystems) was added in 10 μl/well aliquots to the plate. Real-time PCR was carried out using a ViiA 7 Real-Time PCR system (Applied Biosystems). The thermal cycling conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The standard curve was generated using a 4-parameter regression curve-fitting program (Genentech). The coefficient of variation (CV) by analysis of variance and *P* value by pairwise correlation were calculated using the JMP software (SAS, Cary, NC, USA). Values shown are mean ± standard deviation. Error bars in figures are standard deviations.

The VEGF immuno-PCR assay used biotinylated mouse monoclonal anti-VEGF 3.5F8 IgG_1 for capture and DNA-labeled anti-VEGF A4.6.1 for detection. Recombinant $VEGF_{165}$ (Genentech) was used as the standard [4]. VEGF concentrations in colon cancer serum samples were measured using sample buffer containing 500 μg/ml mouse IgG instead of 100 μg/ml to further reduce the possibility of nonspecific signals from anti-mouse antibodies. Increasing the mouse IgG concentration in the sample buffer did not affect the assay standard curve range (data not shown). We compared 0.0062, 0.0125, and 0.025 μg/ml DNA-conjugated detection antibody anti-VEGF A4.6.1 and obtained the same standard curve range. The EGFL7 immuno-PCR assay used biotinylated mouse monoclonal IgG_{2b} anti-EGFL7 9B8 (Genentech) for capture and DNA-labeled anti-EGFL7 18F7 for detection. Truncated recombinant EGFL7 (Genentech) consisting of amino acids 1–167 with a methionine at the N-terminus and 6 histidines at the C-terminus was used as the standard. Conversion between pM and pg/ml concentrations was calculated using molecular weights of 19021 and 27462 for the truncated and full-length EGFL7 molecules, respectively. We compared 0.2 and 0.5 μg/ml biotinylated capture antibody anti-EGFL7 9B8 and obtained the same standard curve range.

VEGF and EGFL7 ELISAs

For VEGF ELISA, MaxiSorp plates (384-well, Nunc, Thermo Fisher Scientific, Rochester, NY, USA) were coated with 0.4 μg/ml anti-VEGF antibody 3.5F8 in 50 mM carbonate, pH 9.6 at 4 °C overnight. Plates were washed with 0.05% polysorbate 20 in PBS, pH 7.4, and then blocked with 0.5% BSA, 15 ppm proclin in PBS, pH 7.4. $VEGF_{165}$ standards and samples in twofold serial dilution in assay buffer (0.5% BSA, 0.05% polysorbate 20, 15 ppm proclin, 0.25% CHAPS, 5 mM EDTA, and 0.35 N NaCl in PBS, pH 7.4) containing 0.5 mg/ml mouse IgG (EquiTech-Bio, Kerrville, TX) were added and incubated for 2 h. Bound VEGF was detected using 0.15 μg/ml biotinylated anti-VEGF antibody A4.6.1 in assay buffer containing

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