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Research paper

Development of a sandwich enzyme-linked immunosorbent assay for the detection of CD44v3 using exon v3- and v6-specific monoclonal antibody pairs

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ABSTRACT

It has been suggested that soluble CD44 levels in cancer patient sera may be closely associated with tumor progression and metastasis. However, to date, there has been limited methodology for detecting the soluble CD44 variant 3 isoform (CD44v3). Herein, using phage display technology, we isolated monoclonal antibodies specific to exon v3 or v6 of CD44 (CD44-exonv3 or CD44-exonv6) from a human synthetic antibody library. We also confirmed the specificity of antibody binding to CD44-exonv3 or -exonv6. Label-free kinetic analysis using the Octet biolayer interferometry system showed that the K_d values of the *anti*-CD44-exonv3 and *anti*-CD44-exonv6 antibodies for CD44v3–10 are approximately 1.1 nM and 1.5 nM, respectively. Finally, we developed a sandwich enzyme-linked immunosorbent assay (ELISA) using the *anti*-CD44-exonv3 and *anti*-CD44-exonv6 antibody pairs. The minimum detection limit of the assay was 6.2 ng/ml CD44v3–10 and the linear range was up to 125 ng/ml. Intra- and inter-assay coefficients of variation were 2.2% and 2.9%, respectively. The intra- and inter-assay recoveries were 99.3% and 105.3%, respectively. Taken together, these results suggest that this novel sandwich ELISA using the *anti*-CD44-exonv3 and *anti*-CD44-exonv6 antibody pairs will be useful for the detection of soluble CD44v3 in cancer patient sera.

1. Introduction

Cancer is the second leading cause of death worldwide (Itzhak, 2010). Despite recent remarkable advances in cancer therapeutic intervention, the early detection of cancers is still important for improving the clinical outcome of cancer patients. However, due to the limited methodology available for early diagnosis, increasing attention has been paid to the development of simple, accurate, and sensitive diagnostic tools for the comprehensive detection of biomarkers that are closely associated with tumor progression and metastasis.

CD44 represents a family of class I transmembrane glycoproteins that are expressed by a wide variety of cells (Stauder et al., 1995; Ni et al., 2002; Forster-Horvath et al., 2004; Song et al., 2005; Reategui et al., 2006). Multiple CD44 isoforms arise from extensive alternative splicing that most often involves the tandem insertion of sequences encoded by variant exons v1 to v10 into the membrane-proximal region of the extracellular domain (Ponta et al., 2003). The CD44 proteins participate in the regulation of a number of diverse cellular processes, including cell proliferation, differentiation, survival, and motility (Legg et al., 2002; Orian-Rousseau, 2010). In pathological conditions, such as tumor progression and metastasis, CD44 stimulates tumor cell proliferation, motility, and invasiveness (Gunthert et al., 1991; Seiter et al., 1993; Hsieh et al., 1999; Forster-Horvath et al., 2004; Marhaba and Zoller, 2004). It has been also suggested that dysregulated CD44 expression is a diagnostic or prognostic marker in various types of cancer (Stauder et al., 1995; Yokota et al., 1999; Wielenga et al., 2000; Naor et al., 2002; Shah et al., 2012; Luo et al., 2014).

CD44v3 is the CD44 isoform containing the alternatively spliced exon v3. Recent investigations have suggested a possible association between CD44v3 and tumor progression. For example, CD44v3 forms complexes with heparin-binding epidermal growth factor-like growth factor, basic fibroblast growth factor, vascular endothelial growth factor, and hepatocyte growth factor (Bennett et al., 1995; Orian-Rousseau, 2010). CD44v3 binds to the oncogene Her2/Neu, which is expressed in ovarian cancer, and also plays a key role in tumor angiogenesis (Bourguignon et al., 1997; Forster-Horvath et al., 2004). Furthermore, several reports have demonstrated the importance of CD44v3 as a

Abbreviations: Ab, antibody; CD44v3, soluble CD44 variant 3 isoforms; CD44-exonv3, exon v3 of CD44; CD44-exonv6, exon v6 of CD44; ELISA, enzyme-linked immunosorbent assay; HEK293F, human embryonic kidney 293F; HRP, horseradish peroxidase; Ig, immunoglobulin; IgG, immunoglobulin G; scFv, single-chain variable-fragment.

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0022-1759/

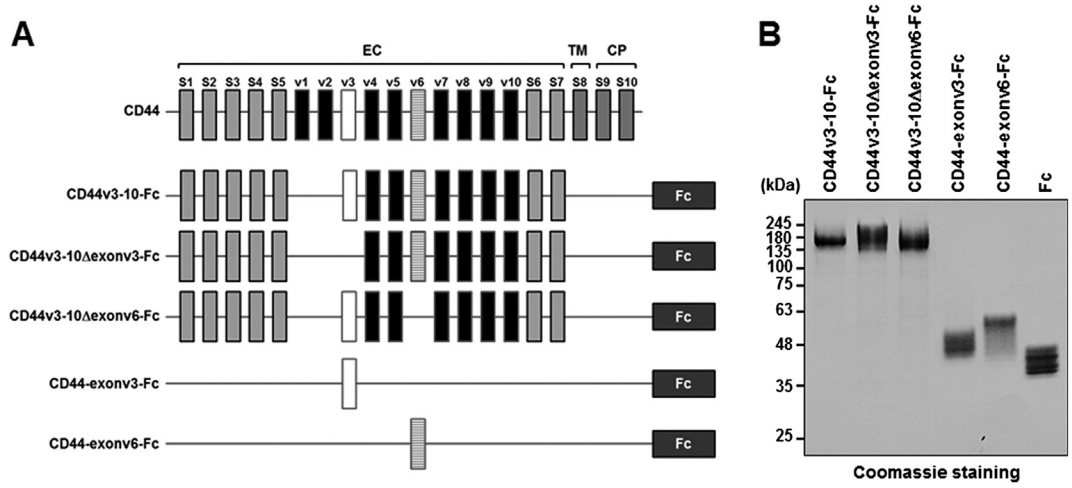

Fig. 1. Expression and purification of wild-type CD44v3–10, exon deletion mutants, and individual exons. (A) Schematic diagram of wild-type CD44v3–10, exon deletion mutants, and individual exons as Fc fusion proteins. EC, TM, and CP denote the CD44 extracellular, transmembrane, and cytosolic domains, respectively. (B) Following the expression of Fc fusion proteins in HEK293F cells and purification using protein A Sepharose affinity chromatography, protein purity was estimated by SDS-PAGE and Coomassie staining.

diagnostic or prognostic marker of head and neck cancers and colorectal cancers (Kuniyasu et al., 2001; Kuniyasu et al., 2002; Reategui et al., 2006). However, despite the strong implication of CD44v3 in cancer, to date, no detection tools are available to measure soluble CD44v3 in human patient sera.

In this study, using phage display technology, we isolated *anti*-CD44-exonv3 and *anti*-CD44-exonv6 monoclonal antibodies from a human synthetic antibody library. Those antibodies identified as specifically targeting CD44-exonv3 or CD44-exonv6 and having nanomolar affinity for CD44v3–10 were further characterized by sandwich enzyme-linked immunosorbent assay (ELISA), which can sensitively, accurately, and reliably detect soluble CD44v3. In summary, this study characterizes specific *anti*-CD44-exonv3 and *anti*-CD44-exonv6 monoclonal antibodies that could potentially be developed for use as serum CD44v3-detection diagnostics for cancer patients.

2. Materials and methods

2.1. Cell culture

Human embryonic kidney 293F (HEK293F) cells were maintained in Freestyle™ 293 expression medium (Invitrogen/Life Technologies, Carlsbad, CA, USA) supplemented with 1% (v/v) penicillin/streptomycin (Gibco, Grand Island, NY, USA) in a humidified Multitron incubation shaker (Infors HT, Basel, Switzerland) at 37 °C in 8% CO_2.

2.2. Construction and preparation of Fc fusion proteins

The DNA sequences encoding CD44v3–10 (amino acids 21–606), CD44-exonv3 (amino acids 243–284), CD44-exonv6 (amino acids 359–401) were amplified by polymerase chain reaction (PCR) using

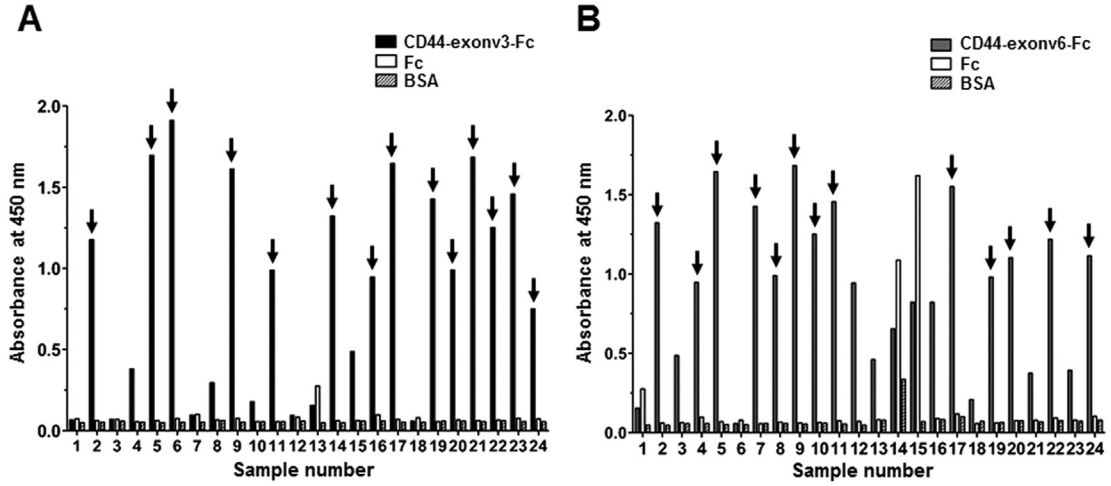

Fig. 2. Isolation of CD44-exonv3- and CD44-exonv6-specific scFvs. A synthetic human scFv antibody library was precleared of Fc binders and screened by biopanning with recombinant CD44-exonv3-Fc or CD44-exonv6-Fc. Twenty-four scFv phage clones (1–24) specific for CD44-exonv3-Fc (A) or CD44-exonv6-Fc (B) were randomly selected and the supernatant was analyzed by phage ELISA. ScFv clone reactivity to CD44-exonv3-Fc or CD44-exonv6-Fc was assayed by determining the absorbance at 450 nm. Arrows indicate scFv clones that are strongly and specifically reactive to CD4-exonv3-Fc (black bars) or CD44-exonv6-Fc (dark gray bars), but not to Fc (white bars). BSA (white hatched bars) was used as a background control.

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