H6D Polymorphism Specific Detection of NAG1 in Human Serum

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ABSTRACT

The nonsteroidal anti-inflammatory drug-activated gene (NAG1), also referred to as GDF-15 or MIC-1, is a member of the TGF-β superfamily of cytokines. NAG1 is implicated in prostate cancer, and levels of NAG1 protein in the serum of patients with metastatic prostate carcinomas are significantly higher than those from patients with breast and colorectal carcinomas. A correlation of the most common and well-characterized position 6 histidine-to-aspartate (H6D) polymorphism is associated with sporadic and familial cases of PCa, and the allelic H6D variation of NAG1 is an independent predictor of the presence of metastasis. The lack of widely available commercial reagents to measure NAG1 protein concentration and detect serum protein variants compels the development of additional antibodies and a highly sensitive ELISA for NAG1 protein. We have undertaken production of a novel NAG1 serum assay allowing for the detection of total NAG1 serum levels. We have produced multiple antibodies that allow for detection of total NAG and that can distinguish between the His6 and Asp6 variants. Additionally we have produced recombinant NAG1 protein standards. The goal is to create a commercially available ELISA with a low background and high signal-to-noise ratio that is capable of detecting serum NAG1 at concentrations of 25 pg/mL or lower.

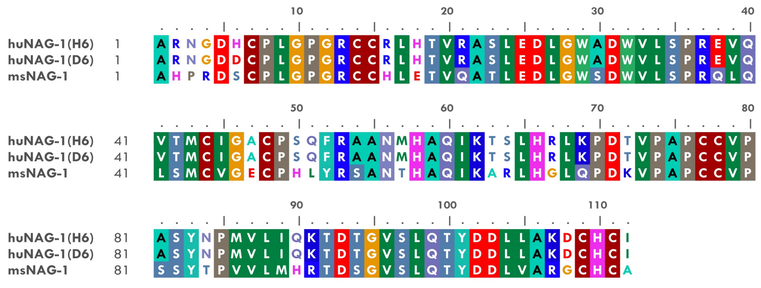
INTRODUCTION

The NSAID-activated gene 1 protein (NAG1) is a divergent member of the transforming growth factor-β (TGF- β) superfamily. It has been reported using several names, including: prostate-derived factor), growth differentiation factor 15 (GDF15), and macrophage inhibitory compound 1 (MIC1). NAG1 has emerged as a potential serum biomarker for several biological conditions. High levels of NAG1 are detected in sera, amniotic fluid and placental extracts during pregnancy. NAG1 is also present at elevated levels in sera from patients diagnosed with prostate cancer [1] and pancreatic cancer [2].

The NAG1 protein is synthesized as an immature 308-amino acid polypeptide composed of 3 sections; a 29-amino acid secretion signal, a 167-amino acid propeptide, and a 112-amino acid mature protein. The propeptide is cleaved at a canonical RXXR furin-like cleavage site to produce the mature 112 amino acid mature protein. Each mature monomer has 3 intramolecular disulfide bonds, and active NAG1 is secreted as a disulfide crosslinked homodimer [3]. A GC polymorphism exists among the population that switches the mature NAG1 position 6 histidine (H) to aspartic acid (D) [3,4] . An epidemiology study using an antibody-based approach determined that 54% of individuals were homologous for histidine at position 6 of the NAG1 protein (HH), 39% were heterologous with the aspartate substitution of histidine (HD), and 7% of the population were homologous with two aspartic acids (DD allele) [4]. Two separate epidemiology studies showed a decreased risk of both sporadic and familial prostate cancer associated with the more uncommon D at position 6 of the mature protein, along with an association between the D6 polymorphism and reduced disease-specific survival among prostate cancer patients [5,6].

The NAG1 H6D polymorphism represents a novel serum biomarker for cancer detection, therapeutic monitoring and epidemiology studies. Currently the only commercially available antiNAG1 antibodies are specific to the NAG1 C-terminal region which is insufficient to develop an ELISA to measure NAG1 serum levels and to detect the levels of NAG1 H and D variants. Additionally researchers using a mouse model require mouse NAG1 specific antibodies. We are generating a panel of NAG antibodies sufficient to produce a highly sensitive ELISA kit to measure total NAG1 and levels of H6 and D6 NAG1 variants in serum. The availability of a NAG1 diagnostic ELISA kit will facilitate the further investigation of this import protein as it relates to a number of diseases, conditions and animal model systems.

MATURE NAG SEQUENCE HUMAN & MOUSE



Alignment of the mature human NAG1 H and D protein variants and mouse NAG1. Residues are colored by property, cyan is hydrophobic, red is negatively charged, blue is positively charged and grey is neutral. Conserved residues are highlighted. The H6D human NAG1 polymorphism is outlined in a blue box and marked below with a blue diamond.

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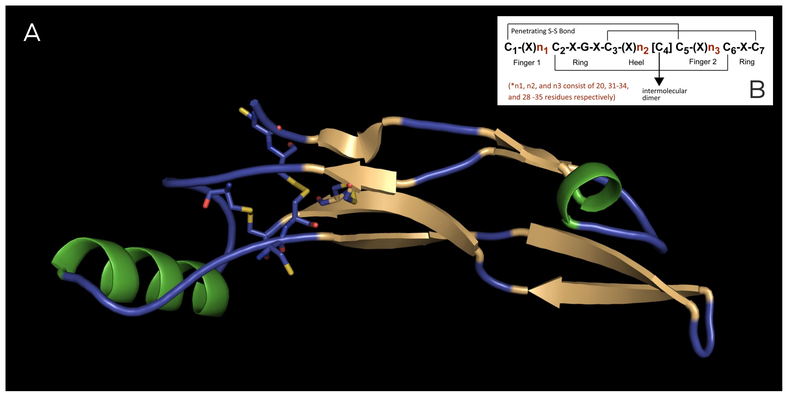
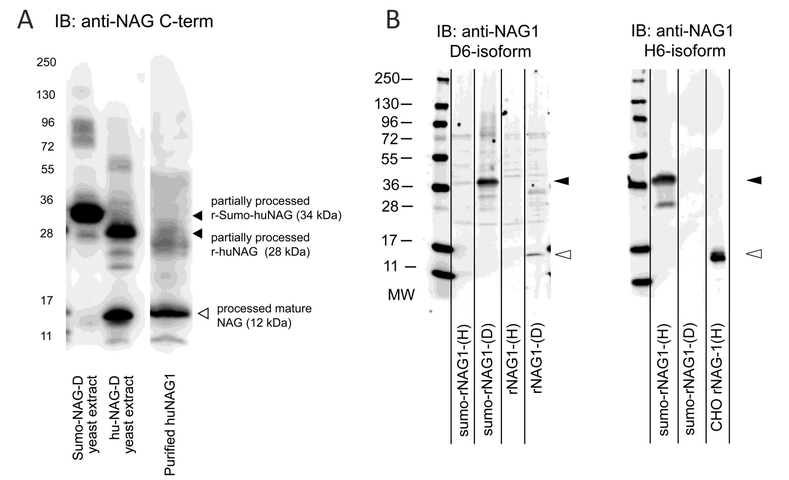
MOLECULAR MODEL OF HUMAN NAG1



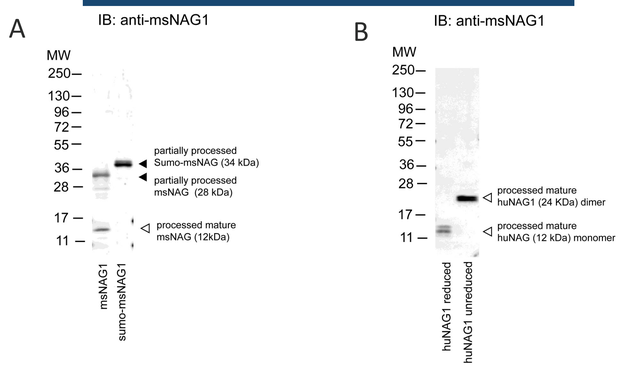
Molecular model of NAG1. NAG1 is a member of the TGF-β family of cytokines. A, model of NAG1 showing the expected cysteine knot motif and three intramolecular disulfide bonds. Blue arrow indicates the position of the NAG1 H6D polymorphism. The model was produced using the Swiss-model program (http://swissmodel.expasy.org/) [7], where the structure of conserved TGF-β superfamily member BMP-2 was used as a template. B. The canonical TGF-ß monomer contains a highly conserved 7-cysteine domain in the C-terminal region. One cysteine [C4] residue is used for inter-chain disulfide bridging, and the others [C1-C5, C2-C6, and C3-C7] are involved in an intramolecular ring formation. This cysteine-knot configuration is a folding motif that forces exposure of hydrophobic residues to the aqueous surrounding, and prevents the molecule from assuming a globular protein structure, resulting in highly stable dimeric protein with a butterfly-shape structure.

POLYCLONAL ANTIBODIES SPECIFIC FOR HUMAN NAG1 & H & D VARIANTS



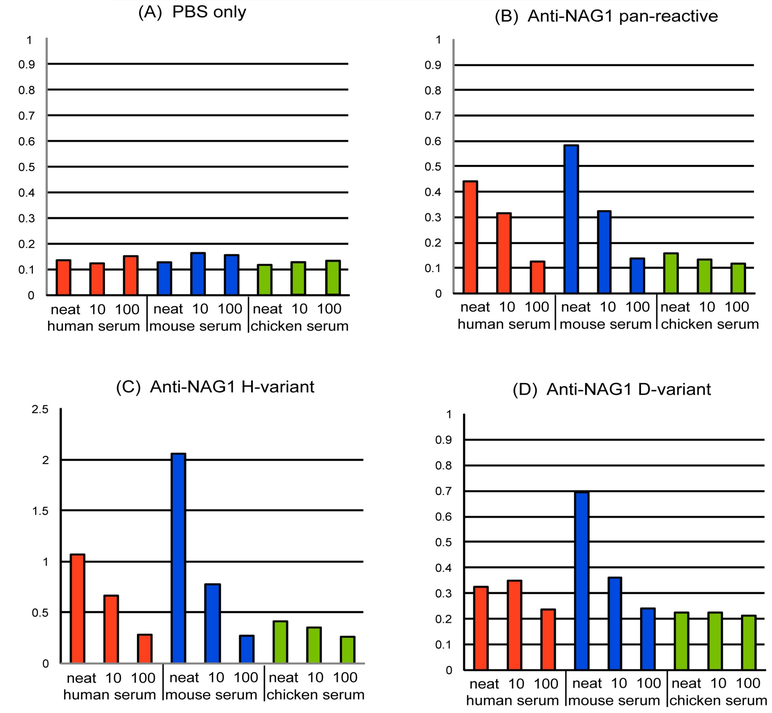
Western blot data for polyclonal anti human NAG1. Polyclonal anti-human NAG1 (C-terminal epitope specific), and polyclonal (H6) and (D6) variant specific antibodies were tested for specificity for NAG1. Recombinant NAG1 (H6) variant or NAG1 (D6) variant protein were independently expressed in Pichia pastoris. The crude mixture of yeast protein containing either NAG1(H) or NAG1(D) was separated in SDS-PAGE and blotted to nitrocellulose. The blots were probed with each of the 3 anti-NAG1 antibodies; anti-NAG1 (C-term), anti-NAG1 (H) or anti-NAG1 (D). A. Anti-NAG1 (C-term) antibody detects recombinant NAG1 expressed in yeast as either a sumo-NAG1 fusion or as wt-NAG1 as indicated by arrows. Additionally the anti-NAG1 (C-term) antibody detects wt-NAG produced in CHO cells (sourced from R&D systems). B. Left. Anti-NAG1 (H) is able to detect recombinant NAG1 (H) but does not detect NAG1 (D). Right; In contrast the anti-NAG1 (D) only detects recombinant NAG1 (D) but does not detect NAG1 (H). Black arrows indicate immature NAG1, open arrow indicates mature NAG1.

POLYCLONAL ANTIBODIES SPECIFIC FOR MOUSE NAG1



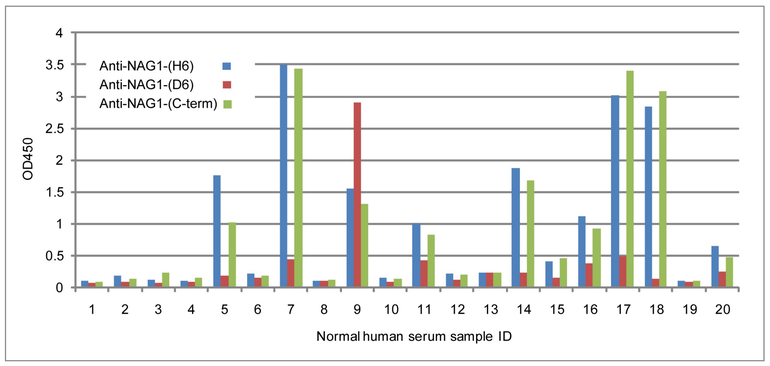
Western blot for anti mouse NAG1. Polyclonal anti-mouse NAG1 (C-terminal epitope specific) antibody was produced by repeated immunizations with a synthetic peptide. Recombinant mouse NAG1 expressed in Pichia pastoris and human NAG1 expressed in CHO cells were separated by SDS-PAGE and blotted to nitrocellulose. The blots were probed with anti-mouse NAG1 (C-term) antibody. A. Anti-msNAG1 (C-term) antibody detects recombinant msNAG1 as either a sumo-msNAG1 fusion or as wt-msNAG1 as indicated by arrows. B. Anti-msNAG1 (C-term) antibody also detects huNAG1 in BOTH the monomeric and dimeric forms. Black arrows indicate immature NAG1, open arrow indicates mature NAG1.

NAG1 ELISA CAPTURE ASSAY USING HUMAN & MOUSE SERUM



ELISA sandwich NAG1 capture assay. In this assay NAG1 was captured from human or mouse serum by various anti-NAG1 N-terminal specific polyclonal antibodies. Detection was performed using HRP-conjugated anti-NAG1 (C-term) antibody. A 96-well microplate was coated with 100 µLof rabbit anti-NAG1 N-term polyclonal (pan-reactive to both the D6 and H6 variants) antibody (panel B), or with anti-NAG1(H) (panel C) or anti-NAG1(D) (panel D) specific antibodies at concentration 1 µg/mL. PBS solution without antibody was used as a negative control (panel A). After overnight incubation of capture antibody, the wells were blocked and independent capture experiments using serum from human, mouse or chicken were performed. 10-fold serial dilutions of normal serum, starting with undiluted serum (neat) were applied and incubated for 1 h at 37 °C. After washing, HRP conjugated anti-NAG1 (C-term) antibody was added (100 µL/well at 1µg/mL) and the plate was further incubated at 37°C for 1 h. Following washing, HRP substrate was added to the plate for 30 min and reaction was stopped by 1 M H2SO4 and measured at 450nm. This demonstrates that the antibodies can detect NAG1 protein in human and mouse serum.

PILOT STUDY: NAG1 POLYMORPHISM IN HUMAN SERUM



Capture ELISA for differential detection of NAG1 H6D in normal human serum. A 96-well microplate was loaded with 3 different anti-NAG1 capture antibodies by coating with 100 µL of either anti-NAG1 (C-term), or anti-NAG1 (H), or anti-NAG1 (D) variant antibodies. Serum from 20 random normal adults was applied to wells for binding of NAG1. After washes, detection of bound NAG1 occurred using HRP conjugated anti-NAG1 (C-term). Although preliminary, the assay results indicate that the anti-NAG1 (C-term) capture antibody bound varying levels of NAG1 in serum samples (i.e. samples 3, 5 and 7). These results also suggest that the anti-NAG1 (H) and anti-NAG1 (D) variant antibodies are able to detect NAG1 polymorphisms in human serum samples. By example, samples 7, 17 and 18 show elevated levels of reactivity by anti-NAG1 (C-term) and anti-NAG1 (H) but low levels of anti-NAG1 (D) reactivity. In contrast, sample 9 shows elevated levels of anti-NAG1 (D) reactivity and lower levels of anti-NAG1 (H) reactivity.

CONCLUSIONS

We have successfully produced polyclonal antibodies against NAG1/MIC1/GDF15 serum protein reactive to both human and mouse sources. These antibodies that can bind monomeric and dimeric NAG1 and distinguish between the NAG1 H6/D6 polymorphism which has been described as a putative biomarker for prostate and pancreatic cancer and other conditions. Preliminary data for the development of an ELISA assay for the detection of NAG1 in serum, and for the capture and detection of polymorphic variant forms (H6) and (D6) of NAG1 shows promising initial results. Further optimization of assay reagents and the inclusion of newly produced variant specific monoclonal antibodies may result in a validated assay to screen for and profile NAG1 variant forms in human serum samples which may be useful for cancer detection, therapeutic monitoring and epidemiology studies.