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 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 for NAG1 protein quantification or detection serum protein variants compels the development of additional antibodies and a highly sensitive ELISA kit 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 NAG1 and that can distinguish between the H6S 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.

Key References

5. 6. 7. 8. 9. 10.

Introduction

The NAG1 protein is synthesized as an immature 308-amino acid polypeptide, which is cleaved by furin-like proteases to generate a panel of NAG antibodies sufficient to produce a highly sensitive ELISA kit and allows for detection of both the D6 and H6 variants of NAG1. The mature human NAG1 protein contains a highly conserved TGF-β superfamily 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 favors exposure of hydrophilic residues to the aqueous surrounding, and prevents the molecule from assuming a globular protein structure, resulting in highly stable dimeric protein with a butterfly-like shape.

Molecular Model of Human NAG1

Alignment of the mature human NAG1 H and D protein variants and mouse NAG1.

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. B. Anti-NAG1 (H) antibody detects wt-NAG1 produced in CHO cells (recovered 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.

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-NAG1 (C-term) antibody detects recombinant mNAG1 as either a sumo-mNAG1 fusion or as wt-mNAG1 as indicated by arrows. B. Anti-mNAG1 (C-term) antibody also detects nNAG1 in both the mono- and dimeric forms. Black arrows indicate immature NAG1, open arrow indicates mature NAG1.

Polyclonal Antibodies Specific for Human NAG1 and H and D Variants

Polyclonal Antibodies Specific for Mouse NAG1

NAG1 ELISA Capture Assay using Human and Mouse Serum

Pilot Study: NAG1 H6D 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), anti-NAG1 (H), or anti-NAG1 (D) variant antibodies. Serum from 20 random normal adults was applied to wells for binding of NAG1. After washing, 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, 4, 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 against 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.

Polyclonal Antibodies Specific for Mouse NAG1

Conclusions

We have successfully produced polyclonal antibodies against NAG1/MIC1/GDF15 serum protein reactive to both human and mouse sources. These antibodies can bind monomeric and dimeric NAG1 and distinguish between the NAG1 H6D 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 polyclonal variants (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 variants in human serum samples which may be useful for cancer detection, therapeutic monitoring and epidemiology studies.

Key References

5. 6. 7. 8. 9. 10.