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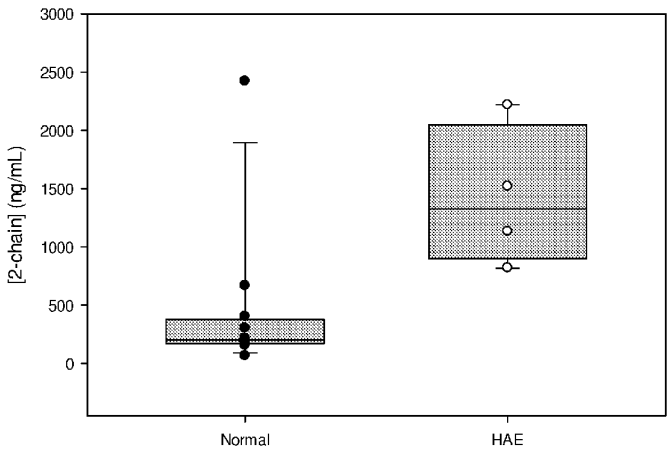
(54) **Title**: IMMUNOASSAY TO DETECT CLEAVED HIGH MOLECULAR WEIGHT KININOGEN

FIG. 9



(57) **Abstract**: The present disclosure provides immunoassay methods of detecting a cleaved high molecular weight kininogen (HMWK) with high sensitivity and specificity and isolated antibodies that specifically bind cleaved HMWK.

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IMMUNOASSAY TO DETECT CLEAVED HIGH MOLECULAR WEIGHT KININOGEN

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Numbers 62/243,505, filed October 19, 2015, and 62/335,311, filed May 12, 2016 under 35 U.S.C. §119, the entire content of each of which is herein incorporated by reference.

BACKGROUND OF PRESENT DISCLOSURE

Kininogens are precursors of kinin, such as bradykinin and kallidin. There are two types of human kininogens, high molecular-weight kininogen (HMWK) and low molecular-weight kininogen (LMWK), which are splicing variants. HMWK acts mainly as a cofactor on coagulation and inflammation and is the preferred substrate for plasma kallikrein (pKal)-mediated bradykinin generation.

Plasma kallikrein (pKal) is the primary bradykinin-generating enzyme in the circulation. The activation of pKal occurs via the contact system which has been linked to disease pathology associated with hereditary angioedema (HAE). pKal cleaves HMWK (a single-chain polypeptide) to produce bradykinin and a cleaved form HMWK, which contains two polypeptide chains held together by a disulfide bond. Cugno et al., Blood (1997) 89:3213-3218.

Cleaved HMWK increased to about 47% of total kininogen during a hereditary angioedema (HAE) attack. Cugno et al., Blood (1997) 89:3213-3218, making it a biomarker for monitoring HAE attack. It is therefore of interest to develop sensitive and reliable assays for detecting the level of cleaved HMWK in biological samples.

SUMMARY OF PRESENT DISCLOSURE

Some aspects of the present disclosure provide an immunoassay for detecting a cleaved high molecular weight kininogen (HMWK) with high sensitivity and specificity. The method comprises (i) providing a support member on which a first agent (*e.g.*, an antibody such as 559B-M004-B04) that specifically binds a cleaved HMWK is attached; (ii) contacting the support member of (i) with a biological sample suspected of containing a cleaved HMWK; (iii) contacting the support member obtained in (ii) with a second agent that binds HMWK, wherein the second agent is conjugated to a label; and (iv) detecting a signal released from the label of the second agent that is bound to the support member, directly or indirectly, to determine the

level of the cleaved HMWK in the biological sample. In some instances, step (ii) may be performed in the presence of $ZnCl_2$.

In some embodiments, prior to step (ii), the support member of (i) is incubated with a blocking buffer.

In some embodiments, the second agent is a polyclonal antibody, a monoclonal antibodies, or a mixture of two or more monoclonal antibodies that bind to HMWK. The two or more monoclonal antibodies in the mixture may bind to different epitopes in HMWK. In some embodiments, the label is a signal releasing agent. In some embodiments, the label is a member of a receptor-ligand pair. In that case, the immunoassay may further comprise, prior to step (iv), contacting the second agent in (iii), which is immobilized on the support member, with the other member of the receptor-ligand pair, wherein the other member is conjugated to a signal releasing agent. In one example, the receptor-ligand pair is biotin and streptavidin.

Another aspect of the present disclosure provides methods for detecting a cleaved high molecular kininogen (HMWK) in a sample, the method comprising (i) contacting a sample suspected of containing a cleaved HMWK with any of the antibodies described herein (*e.g.* 559B-M004-B04); (ii) measuring a complex of the cleaved HMWK and the antibody formed in step (i); and (iii) determining the level of the cleaved HMWK in the sample based on the result of step (ii). In some embodiments, step (i) is performed in the presence of $ZnCl_2$. In some embodiments, step (i) is performed using an enzyme-linked immunosorbent assay (ELISA) or an immunoblotting assay.

In any of the methods described herein, the sample may be a biological sample obtained from a subject (*e.g.*, a human patient), such as a serum sample of a plasma sample. In some embodiments, the method further comprises collecting the sample into an evacuated blood collection tube, which comprises one or more protease inhibitors.

Any of the assay methods (*e.g.*, immunoassays) described herein may be a ELISA assay, a Western blot assay, or lateral flow assay.

In some embodiments, the biological sample is obtained from a subject (e.g., a human patient) having a disease. The assay method may further comprise determining whether the disease is mediated by plasma kallikrein based on the level of the cleaved HMWK, a deviation of the level of the cleaved HMWK in the sample from that of a control sample being indicative that the disease is mediated by plasma kallikrein.

Any of the assay methods described herein may further comprise identifying patients with diseases or disorders mediated by plasma kallikrein, or evaluating the efficacy of a

treatment of the disease or disorder based on the levels of cleaved HMWK. In some embodiments, the method may further comprises administering to the subject an effective amount of a therapeutic agent, such as a plasma kallikrein (pKal) inhibitor, a bradykinin 2 receptor (B2R) inhibitor, and/or a C1 esterase inhibitor, for treating the disorder, if the subject is identified as having the disorder. In some embodiments the pKal inhibitor is an anti-pKal antibody. In some embodiments, the therapeutic agent is lanadelumab, ecallantide, icatibant, or human plasma-derived C1 esterase inhibitor.

In some embodiments, the subject is a human patient who is on a treatment for the disorder, and wherein the method further comprises assessing the efficacy of the treatment based on the level of the cleaved HMWK determining in step (iii), a deviation of the level of the cleaved HMWK in the sample from the subject from that of a control sample being indicative of the treatment efficacy. In some embodiments, the method further comprises identifying a suitable treatment for the subject based on the level of the cleaved HMWK. In some embodiments, the method further comprises identifying the subject as a candidate for a treatment of the disease based on the level of the cleaved HMWK.

In some embodiments, the human patient has a history of the disease (*e.g.*, HAE). In some embodiments, the method further comprises assessing the risk of disease attack in the subject based on the level of the cleaved HMWK, a deviation of the level of the cleaved HMWK in the sample from the subject from that of a control sample being indicative of the risk of disease attack. In some embodiments, the method further comprises administering a therapeutic agent to the subject, if the subject is at risk of disease attack.

In another aspect, a kit is provided for detecting a cleaved high molecular weight kininogen (HMWK), the kit comprising a first agent (*e.g.*, an antibody as described herein) that specifically binds a cleaved HMWK. In some embodiments, the kit further comprises a second agent that binds HMWK, a support member, or both, and optionally instructions for detecting the cleaved HMWK. In some examples, the support member is a 96-well plate.

In another aspect of the disclosure, an isolated antibody is provided, which specifically binds a cleaved high molecular weight kininogen (HMWK). In some embodiments, the antibody binds the same epitope as 559B-M004-B04 or competes against 559B-M004-B04 for binding to the cleaved HMWK. In some embodiments, the antibody comprises the same heavy chain and light chain complementary determining regions as 559B-M004-B04, e.g., the same heavy chain and light variable regions as 559B-M004-B04. In one example, the antibody is 559B-M004-B04.

Any of the antibodies specific to a cleaved HMWK as described herein can be used in a method for detecting a cleaved high molecular kininogen (HMWK) in a sample. Such a method may comprise (i) contacting a sample suspected of containing a cleaved HMWK with the antibody; (ii) measuring a complex of the cleaved HMWK and the antibody formed in step (i); and determining the level of the cleaved HMWK in the sample based on the result of step (ii). In some embodiments, the sample is a biological sample such as a serum sample or a plasma sample obtained from a human subject. The result obtained from this method may be relied on to determine the risk of a subject from whom the sample is obtained for developing a disorder mediated by plasma kallikrein such as HAE. In some instances, step (i) can be performed in the presence of $ZnCl_2$.

Any of the immunoassay methods described herein can be in Western blot format or ELISA format.

In yet another aspect, an isolated antibody is provided that binds both intact high molecular weight kininogen (HMWK) and a cleaved HMWK.

In some embodiments, the antibody that binds both intact and cleaved HMWK does not bind to low molecular weight kininogen (LMWK). In some embodiments, the antibody binds the same epitope as 559B-M0067-E02, 559B-M0039-G07, 559B-M0044-E09, 559B-M0003-C08, 559B-M0039-H06, 559B-M0039-D08, 559B-M0068-C07, 559B-M0021-G11, 559B-M0061-G06, 559B-M0036-G12, 559B-M0042-E06, 559B-M0070-H10, 559B-M0068-D01, or 559B-M0004-E08. In some embodiments, the antibody competes against 559B-M0067-E02, 559B-M0039-G07, 559B-M0044-E09, 559B-M0003-C08, 559B-M0039-H06, 559B-M0039-D08, 559B-M0068-C07, 559B-M0021-G11, 559B-M0061-G06, 559B-M0036-G12, 559B-M0042-E06, 559B-M0070-H10, 559B-M0068-D01, or 559B-M0004-E08 for binding to the intact HMWK and/or the cleaved HMWK.

In some embodiments, the antibody comprising the same heavy chain and light chain CDRs as 559B-M0067-E02, 559B-M0039-G07, 559B-M0044-E09, 559B-M0003-C08, 559B-M0039-H06, 559B-M0039-D08, 559B-M0068-C07, 559B-M0021-G11, 559B-M0061-G06, 559B-M0036-G12, 559B-M0042-E06, 559B-M0070-H10, 559B-M0068-D01, or 559B-M0004-E08. In some examples, the antibody is selected from the group consisting of 559B-M0067-E02, 559B-M0039-G07, 559B-M0044-E09, 559B-M0003-C08, 559B-M0039-H06, 559B-M0039-D08, 559B-M0068-C07, 559B-M0021-G11, 559B-M0061-G06, 559B-M0036-G12, 559B-M0042-E06, 559B-M0070-H10, 559B-M0068-D01, and 559B-M0004-E08.

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