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TOPIC HIGHLIGHT

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Helicobacter pylori neutrophil-activating protein: From molecular pathogenesis to clinical applications

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Abstract

Helicobacter pylori (*H. pylori*) neutrophil-activating protein (HP-NAP) was originally identified as a virulence factor of *H. pylori* for its ability to activate neutrophils to generate respiratory burst by releasing reactive oxygen species. Later on, HP-NAP was also found to be involved in the protection of *H. pylori* from DNA damage, supporting the survival of *H. pylori* under oxidative stress. This protein is highly conserved and expressed by virtually all clinical isolates of *H. pylori*. The majority of patients infected with *H. pylori* produced antibodies specific for HP-NAP, suggesting its important role in immunity. In addition to acting as a pathogenic factor by activating the innate immunity through a wide range of human leukocytes, including neutrophils, monocytes, and mast cells, HP-NAP also mediates adaptive immunity through the induction of T helper cell type 1 responses. The pro-inflammatory and immunomodulatory properties of HP-NAP not only make it play an important role in disease pathogenesis but also make it a potential candidate for clinical use. Even though there is no convincing evidence to link HP-NAP to a disease outcome, recent findings supporting the pathogenic role of HP-NAP will be reviewed. In addition, the potential clinical applications of HP-NAP in vaccine development, clinical diagnosis, and drug development will be discussed.

Key words: *Helicobacter pylori*; *Helicobacter pylori* neutrophil-activating protein; Clinical application; Vaccine; Diagnosis; Drug development; Immunotherapy; Immunomodulation; T helper cell type 1/2

Core tip: *Helicobacter pylori* (*H. pylori*) neutrophil-activating protein (HP-NAP) acts as a virulence factor to play a pathogenic role in *H. pylori* infection. However, the unique immune properties and biological function of HP-NAP make it a potential candidate for clinical applications, including vaccine development, clinical diagnosis, and drug development.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) was first isolated in 1982 from human gastric biopsy[1]. Today, it is a well-recognized pathogen that chronically infects up to approximately half of the world's human population[2,3]. Infection with *H. pylori* causes chronic gastritis and peptic ulcer disease. Also, chronic *H. pylori* infection was found to be associated with an increased risk of gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma[4]. In 1994, *H. pylori* was classified as a carcino-

gen in humans by the International Agency for Research on Cancer of the World Health Organization. In order to develop novel and more effective strategies in the prevention and treatment of *H. pylori* infection to overcome the increasing failure of standard triple therapy for *H. pylori*, great efforts have been made to identify the virulence factors contributing to the pathogenesis of *H. pylori* infection. Several virulence factors, such as urease, vacuolating cytotoxin (VacA), cytotoxin-associated gene A (CagA), and neutrophil-activating protein (NAP), are well characterized for their roles in bacterial colonization and gastric inflammation during *H. pylori* infection[5,6]. These factors are also immunodominant antigens of *H. pylori*[7]. Among them, *H. pylori* neutrophil-activating protein (HP-NAP) might play a crucial role in *H. pylori*-induced gastric inflammation due to its ability to attract and activate neutrophils.

PATHOGENIC ROLES OF HP-NAP

HP-NAP was first identified from the water extract of *H. pylori* for its ability to stimulate the production of reactive oxygen species (ROS) in neutrophils and promote neutrophil adhesion to endothelial cells[8]. This protein is mainly localized in the bacterial cytosol[9] and may be released upon autolysis. In addition to direct interaction with neutrophil glycosphingolipids[10], HP-NAP might act as an adhesin to bind specifically to sulfated carbohydrates on mucin[11]. HP-NAP is a spherical dodecameric protein consisting of twelve identical monomers[12,13]. Each monomer is a four-α-helix bundle protein with a molecular weight of 17 kDa[12,13]. According to the sequence analysis, HP-NAP belongs to the family of DNA-protecting proteins from starved cells (Dps)[12,14], whose structures are similar to those of the family of ferritin proteins. HP-NAP, just like ferritins and Dps-like proteins, is capable of binding iron[12]. This protein might originally have been an iron-binding and/or iron-regulated protein and later evolved as a pro-inflammatory molecule[5]. However, whether the iron-binding ability of HP-NAP is related to the pathogenesis of *H. pylori* infection is not clear. In addition, a large number of positively charged residues are present on the surface of HP-NAP[13]. This specific characteristic of HP-NAP might account for its unique ability in activating human leukocytes to stimulate the immune response during *H. pylori* infection.

Role of HP-NAP in bacterial protection and survival

To establish a persistent infection, *H. pylori* must survive and colonize in the harsh environment of the stomach. HP-NAP has been reported to participate in the adherence of *H. pylori* to host cells. This protein may expose on the surface of bacterial outer membrane and act as an adhesion molecule by binding to mucin to mediate *H. pylori* adhesion to gastric mucosa[11]. In a study using a *napA* knock-out mutant *H. pylori* strain, HP-NAP was proposed to facilitate sialic acid-binding adhesin (SabA)-mediated binding of sialylated antigens on the host cell surface[15]. An additional study further showed that this *napA* knock-out mutant strain is more sensitive to oxidative stress[16]. The concentration of free iron ions and the degree of DNA damage are much higher in the *napA* knock-out mutant strain than those in the wild-type strain[16]. One mode in which HP-NAP protects DNA from damage may be due to its ability to bind DNA and thus to prevent DNA from attack by free radicals. Interestingly, only the iron-loaded HP-NAP, not apo-HP-NAP, was able to bind DNA[16]. However, a later study reported that iron loading did not affect the ability of HP-NAP to bind DNA[17]. Further analysis using gel mobility assays and atomic force microscopy imaging in that report showed that the positively charged protein surface of HP-NAP was mainly responsible for binding and condensing DNA[17], which is quite different from the DNA binding by the other Dps proteins. As for *E. coli* Dps, its positively charged N-terminus is responsible for the binding of DNA[18]. The other mode of DNA protection by HP-NAP might be due to its iron-sequestering ability to reduce the oxidative stress produced in ferrous ion-mediated Fenton reactions[19]. In an animal study to investigate *H. pylori* colonization in mice infected with both the wild-type and *napA* mutant strains, the degree of survival of the *napA* mutant strain was found to be much lower than that of the wild-type strain[16]. Thus, HP-NAP plays a role in bacterial protection and survival primarily by preventing DNA damage from oxidative stress and probably also by facilitating the adherence of *H. pylori* for its colonization.

Role of HP-NAP in host inflammation

The hallmark of chronic gastritis caused by *H. pylori* infection is infiltration of neutrophils and mononuclear cells into gastric mucosa. In patients infected with *H. pylori*, the degree of gastric mucosal damage is associated with an increase in neutrophil infiltration[20,21]. Infiltrating leukocytes synthesize and secrete inflammatory mediators to recruit and activate additional leukocytes to the injured mucosa, thus amplifying the pathogenic signals which lead to more severe damage of the stomach. HP-NAP plays a critical role in recruiting neutrophils to inflamed mucosal tissue to trigger the gastric inflammatory response during *H. pylori* infection. This protein activates neutrophils by stimulating the production of ROS and secretion of myeloperoxidase[8,22]. HP-NAP also induces chemotaxis and upregulates the expression of β2 integrin (CD18) in both neutrophils and monocytes[23]. In an *in vivo* study using intravital microscopy analysis, HP-NAP has been shown to cross the endothelia to promote neutrophil adhesion to endothelia of rat mesenteric microvessels[24]. This HP-NAP-induced adhesion depends on the acquisition of a high affinity state of β2 integrin on the plasma membrane of neutrophils[24]. In another study using a transwell chamber system, live *H. pylori* induced significantly increased transendothelial migration of neutrophils, but formalin-killed bacteria did not[25]. Also, the transendo-

thelial migration of neutrophils induced by the culture filtrate of the *H. pylori* isogenic HP-NAP deletion mutant is much less than that induced by the culture filtrate of the wild-type strain[25]. These findings support the idea that HP-NAP could be released or secreted from live *H. pylori* to contribute to the recruitment of neutrophils to the gastric mucosa. Once the released HP-NAP encounters monocytes, this bacterial protein could promote the survival of these cells by stimulating their secretion of the endogenous mediator, IL-1β[26]. In the presence of monocytes, HP-NAP could further increase the lifespan of neutrophils[26]. Thus, HP-NAP may play roles in triggering and maintaining gastric inflammation through prolonged activation of myeloid cells.

In addition to the stimulation of ROS production in neutrophils and monocytes[8,23], HP-NAP induces the synthesis and release of interleukin-8 (IL-8, also termed CXCL8), macrophage inflammatory protein 1 alpha (MIP-1α, also termed CCL3), and MIP-1β, also termed CCL4, by neutrophils[24]; the production of tissue factor (TF) and plasminogen activator inhibitor-2 (PAI-2) by human blood mononuclear cells (MNCs)[27]; the secretion of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and IL-8 by monocytes[28]; and the release of β-hexosaminidase and IL-6 by mast cells[29]. HP-NAP-induced production of TF and PAI-2 by human blood MNCs might also contribute to the development of gastritis and tissue damage during *H. pylori* infection since tissue healing could be inhibited due to the procoagulant and antifibrinolytic activities of TF and PAI-2[27]. In addition, TNF-α and interferon gamma (IFN-γ) have been shown to prime human neutrophils to potentiate the effect of HP-NAP on ROS production[23]. HP-NAP and these cytokines may also act synergistically to induce the production of ROS. The production of ROS and the above mentioned cytokines and/or chemokines induced by HP-NAP could act as a pro-inflammatory signal to activate inflammation and oxidative damage of stomach mucosa, which would promote the growth of *H. pylori* by means of nutrient factors released from the inflamed tissue.

The ratio of pro-inflammatory to anti-inflammatory cytokines produced by the host in response to *H. pylori* infection could determine the outcome of *H. pylori*-associated pathology. The T helper (Th) cells, a type of T-lymphocytes, produce enormous amounts of these two types of cytokines. The helper type I (Th1) cytokines produced by Th1 cells produce a pro-inflammatory response to stimulate the phagocytosis and destruction of microbial pathogens, whereas Th2 cytokines produced by Th2 cells produce an anti-inflammatory response to avoid the extensive inflammatory tissue injury and to promote allergic responses. The predominance of Th1 and Th2 responses not only provides the strategy for host protection against pathogens but also determines the pathological outcomes of a disease[30]. It has been reported that acute infection with *H. pylori* induces a Th1 dominant response, which is found to be associated with gastric pathology in *H. pylori*-infected humans[31,32]. Whether HP-NAP plays a role in this cell-mediated immunity has also been studied. HP-NAP stimulates neutrophils and monocytes to express IL-12, which favors Th1 responses[28]. In addition, this protein induces monocytes to express IL-23 and to differentiate to dendritic cells[28] and stimulates human macrophages to express the major histocompatibility complex class Ⅱ[33]. Therefore, HP-NAP should be capable of promoting the induction of Th1 responses. Indeed, the addition of HP-NAP into a culture of antigen-induced T cell lines resulted in a significant shift from a polarized Th2 to cytotoxic Th1 response as shown by the increased number of IFN-γ-producing T cells and the decreased number of IL-4-secreting T cells[28]. Thus, HP-NAP might also contribute to the pathogenesis of *H. pylori* by inducing the Th1 response. The models of how HP-NAP exerts its pathological effects by promoting leukocyte recruitment, secreting pro-inflammatory cytokines, and subsequently stimulating its immunomodulating activity are well illustrated in the other literature reviews[34,35].

The molecular mechanisms by which HP-NAP stimulates ROS production by neutrophils have been studied extensively. ROS is produced by the activation of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase on the plasma membrane. The signal pathway involves pertussis toxin-sensitive heterotrimeric G protein, phosphatidylinositol 3-kinase (PI3-K), Src family tyrosine kinase, and an elevation of cytosolic calcium level[23]. Extracellular regulated kinase (ERK) and p38-mitogen-activated protein kinase (p38-MAPK) are also important in eliciting the HP-NAP-induced respiratory burst, adhesion, and chemotaxis by neutrophils[36]. The involvement of heterotrimeric G protein in the signaling events induced by HP-NAP indicates that the receptor of HP-NAP should be a G protein-coupled receptor (GPCR). However, the identity of this receptor awaits further investigation. HP-NAP-induced production of TF and PAI-2 by human blood MNCs requires protein kinase C, protein tyrosine kinase, and nuclear factor-kappa B (NF-κB), but not NADPH oxidase[27]. It is not clear whether the production of TF and PAI-2 is also mediated by GPCRs. In addition to the unidentified GPCR, Toll-like receptor 2 (TLR2) has been shown to be the receptor of HP-NAP. A study using NF-κB luciferase reporter assay indicates that HP-NAP induced the NF-κB activation only in HEK293 cells expressing TLR2 but not other TLRs[28]. The engagement of TLR2 seems to be related to HP-NAP-induced production of cytokines by monocytes. In a study using a TLR2-blocking antibody, TLR2 was shown to be involved in HP-NAP-stimulated release of IL-6 in splenocytes[37]. The identification of inhibitors to specifically block the interaction between HP-NAP and its receptor might provide an alternative approach for the treatment of *H. pylori* infection.

Disease associations with HP-NAP

Infection with *H. pylori* is associated with gastritis, peptic ulcer disease, gastric adenocarcinoma, and gastric

MALT lymphoma[4]. A possible association of *H. pylori* infection with extragastroduodenal diseases, including iron deficiency anemia (IDA), has also been reported[38]. Investigation of whether and how HP-NAP is involved in these reported associations might lead to a better understanding of the role of HP-NAP in the pathogenesis of diseases caused by *H. pylori*. For gastroduodenal diseases, only one study showed that the level of HP-NAP-specific antibodies in sera from *H. pylori*-infected patients with gastric cancer was significantly higher than that from patients with chronic gastritis[39]. No report has shown the direct association of HP-NAP with *H. pylori*-induced gastric inflammation in patients. However, NapA of *Borrelia burgdorferi* (*B. burgdorferi*), a member of the Dps-like protein family, has been shown to promote the recruitment of both neutrophils and T lymphocytes and the Th17 cell-mediated inflammatory responses in *B. burgdorferi*-induced arthritis[40]. Of interest, both HP-NAP from *H. pylori* and NapA from *B. burgdorferi* activate TLR2 and elicit innate but slightly different T cell pro-inflammatory responses[28,40]. Thus, HP-NAP should also play roles in inducing chronic inflammation in patients with *H. pylori* infection. As for extragastroduodenal diseases, a positive correlation between polymorphism in HP-NAP and IDA has been reported recently. At amino acid residue No. 70, both serine and threonine were found in the *napA* gene in *H. pylori* strains derived from the infected patients[41]. The frequency of the *napA* gene encoding threonine at amino acid residue No 70 in *H. pylori* derived from patients with IDA was much higher than that from *H. pylori*-infected patients without IDA[41]. The iron-uptake ability of *H. pylori* strains with Thr70-type HP-NAP has also been found to be much higher than that of strains with Ser70-type HP-NAP[41], suggesting that the enhanced ability of iron-uptake by Thr70-type HP-NAP is related to the pathogenesis of IDA. However, biochemical analysis of these two types of HP-NAP is needed for a direct proof of their iron-uptake activities. In addition, HP-NAP has been suggested to contribute to the pathology of anti-aquaporin-4 (anti-AQP4) antibody-related neural damage in Japanese patients with multiple sclerosis (MS) and neuromyelitis optica (NMO) by acting as a systemic inflammatory stimulus targeting neutrophils[42]. This implication is based on the finding of the positive correlation of anti-HP-NAP antibody response with anti-AQP4 in those patients[42]. Hence, more convincing evidence needs to be provided to support the idea that HP-NAP is involved in anti-AQP4 antibody-related neural damage in MS/NMO patients.

CLINICAL APPLICATIONS OF HP-NAP

Because of the unique immune properties and biological functions of HP-NAP, this protein has a range of potential clinical applications, including vaccine development, clinical diagnosis, and drug development. Also, strategies for efficient purification of recombinant HP-NAP have been developed to fulfill the needs for its clinical use[43,44].

Vaccine development

Many efforts have been devoted to the development of vaccines against *H. pylori* in humans for either prophylactic or therapeutic purposes since the discovery of *H. pylori* almost two decades ago. The progress in vaccine development against *H. pylori* has been reviewed elsewhere[45,46]. Through the identification and characterization of the virulence factors of *H. pylori*, the development of a vaccine against *H. pylori* has become possible and feasible in humans. Among these virulence factors, HP-NAP is highly immunogenic in humans[23] and thus has become an attractive candidate antigen for the design of *H. pylori* vaccine. HP-NAP was demonstrated to be effective as a vaccine immunogen in both prophylactic and therapeutic protection against *H. pylori* in animal models. Oral immunization of mice with recombinant HP-NAP protected 80% of animals from *H. pylori* infection[23], supporting that HP-NAP is a protective antigen and a vaccine candidate for *H. pylori* prophylaxis. Furthermore, immunizations with the vaccine containing recombinant HP-NAP and CagA proteins in mice through the mucosal priming followed by systemic boosting had enhanced both local and systemic immune responses to these two *H. pylori* antigens[47]. In another study, intramuscular administration of the multicomponent vaccine containing recombinant HP-NAP, CagA, and VacA proteins formulated with aluminum hydroxide in experimentally *H. pylori*-infected beagle dogs reduced the colonization of *H. pylori* and the severity of gastric pathology in these animals[48]. Therefore, parenteral vaccination with this protein vaccine containing HP-NAP as one of the components might be used as a therapeutic means to eradiate *H. pylori* infection. This same protein vaccine has further been demonstrated to be safe and immunogenic in humans[49]. However, whether such a multicomponent protein vaccine can be used for immunoprophylaxis against *H. pylori* infection in humans needs to be further evaluated.

In addition to being administered as a purified antigen, HP-NAP has also been delivered into mice by live vectors, such as attenuated *Salmonella typhimurium*[50], *Lactococcus lactis*[51], and attenuated measles virus vaccine strains[52]. Positive antibody responses to HP-NAP were detected in the animal sera in all three studies[51-53]. HP-NAP-specific cell-mediated immunity was also stimulated as determined by antigen-specific induction of IFN-γ expression in the study using attenuated measles virus[52]. Recently, a study using live attenuated measles virus expressing HP-NAP-tagged chimeric antigens showed that HP-NAP can act as an immunostimulator to enhance the immunogenicity of poor immunogens[53]. Thus, HP-NAP could play a dual role in vaccine development by acting as either an immunogen in a vaccine against *H. pylori* or an immunoadjuvant in a DNA vaccine.

Clinical diagnosis

There are several clinical tests to diagnose *H. pylori* infection. Although a biopsy check during endoscopy with rapid urease test (RUT), histological examination, and

microbial culture are more reliable for the detection of *H. pylori* infection, noninvasive clinical tests, including ^{13}C-urea breath test (UBT), serological test, and stool antigen test, are more easily accepted by patients[54]. Serological tests that detected anti-*H. pylori* IgG antibody are widely used since they are convenient and economical for both patients and physicians[54]. Common designs of an antibody-based serological test include enzyme-linked immunosorbent assay (ELISA), immunoblot test, and immunochromatographic test. However, the performance of these various assays for the serological test is largely dependent on the nature of the antigens used. A study evaluating the performance of commercially available immunoblot and immunochromatographic tests covering the current infection marker (CIM) and conventional ELISA for the diagnosis of *H. pylori* infection in adult dyspeptic patients showed that immunoblot and immunochromatographic tests with CIM were more specific and accurate than the conventional ELISA[55]. CIM, which was originally identified by screening immunogenic proteins of *H. pylori* from the cDNA Genelab library, acts as an indicator of current infection with *H. pylori*[56]. Whether HP-NAP, which is highly immunogenic in humans[23] and is highly conserved and expressed by virtually all clinical isolates, could be used as one of the CIMs to improve the performance of the test still needs further investigation.

The possibility of using HP-NAP as a target to develop an ELISA for clinical diagnosis of *H. pylori* infection by detection of the antibodies against HP-NAP in humans has been explored. A recombinant HP-NAP-based ELISA has been applied to detect serum antibodies against HP-NAP in *H. pylori*-infected patients. The reported positive rates of HP-NAP antibody production in *H. pylori*-infected patients are 60% (21 out of 35)[23] and 89.4% (135 out of 151)[57]. This discrepancy may be due to the different ethnics groups used for the serological tests in these two studies. It is not sure whether the production rate of HP-NAP-specific antibody is higher in the Chinese population.

In addition, whether HP-NAP can serve as a disease-related biomarker to predict a particular clinical outcome of *H. pylori* infection has also been reported. A Chinese study showed that HP-NAP-specific antibody response was significantly higher in *H. pylori*-infected patients with gastric cancer than that in patients with chronic gastritis[39]. In another study using a proteomic approach based on surface-enhanced laser desorption/ionization-time-of-flight-mass spectrometry, the protein levels of HP-NAP in the *H. pylori* strains isolated from *H. pylori*-infected Colombian patients with gastric cancer were higher than those from patients with duodenal ulcer[58]. Thus, HP-NAP could serve as a biomarker for the development of diagnostic kits to predict the evolution of gastric cancer in *H. pylori*-infected patients. Recently, a quantitative capture ELISA for detection of HP-NAP has been developed and may be used for this purpose. This monoclonal antibody-based immunoassay is highly specific and sensitive for detection of native HP-NAP[59] and can be applied in characterization of the HP-NAP-based vaccine.

Drug development

The standard first-line treatment for *H. pylori* infection is a one week "triple therapy" consisting of proton pump inhibitors such as omeprazole and the antibiotics clarithromycin plus either amoxicillin or metronidazole[60]. However, rising antibiotic resistance in *H. pylori*-related ulcer therapy has lead to the development of new therapeutic strategies, including sequential, bismuth-based quadruple and nonbismuth-based quadruple therapies[61]. Recently, the expression of HP-NAP was found to be induced in *H. pylori* treated with colloidal bismuth subcitrate, a component of the bismuth-based antiulcer drug[62], suggesting that the level of HP-NAP in *H. pylori*-infected patients treated with bismuth-based drugs could be increased, and thus the inflammation might be enhanced in those patients during the treatment.

HP-NAP could act as a target for new drugs against *H. pylori*-induced inflammation[63]. Arabinogalactan proteins (AGPs) extracted from Chios mastic gum (CMG) were found to be able to inhibit HP-NAP-induced neutrophil adhesion to endothelial cells[64]. A further study showed that CMG, a natural product from the plant *Pistacia lentiscus var Chia,* could benefit patients infected with *H. pylori* by inhibiting HP-NAP-induced ROS production in neutrophils[65]. The finding that AGPs bound to specific membrane proteins from human neutrophils suggests that AGPs might interact with the receptor of HP-NAP[65]. However, whether AGP acts as an antagonist of the receptor of HP-NAP needs further investigation. Although blocking the action of HP-NAP might not be able to eradicate *H. pylori* infection, identification of the drugs against the action of HP-NAP should lead to the discovery of novel therapeutic agents for reducing the gastric inflammation in patients infected with *H. pylori*. Since all *H. pylori* strains contain HP-NAP with a high degree of protein sequence homology, HP-NAP should still be a good target for the drug development to alleviate *H. pylori*-related diseases.

In addition to acting as a drug target for the treatment of *H. pylori* infection, recombinant HP-NAP itself could serve as a potential drug candidate in the treatment of allergic diseases and immunotherapy of cancer. HP-NAP has been shown to act as an immune modulating agent to suppress Th2 responses in ovalbumin-induced allergic asthma and *Trichinella spiralis* infection[37,66]. By activating cytotoxic Th1 responses, HP-NAP inhibits the growth of bladder cancer[67]. In addition, expression of secreted HP-NAP by oncolytic measles virus and adenovirus has been shown to enhance the antitumor activity of these viruses in the treatment of metastatic breast cancer and neuroendocrine tumors, respectively[68,69]. Since all these studies were done in mouse models, whether HP-NAP can be applied to treat allergic diseases and certain cancers in humans awaits the results from clinical studies. However, HP-NAP definitely offers a novel therapeutic

approach for these diseases.

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