Exp Oncol 2009
31, 3, 134–139

ORIGINAL CONTRIBUTIONS

Experimental ONCOLOGY

HELICOBACTER PYLORI INFECTION OF GASTRIC CANCER CELLS ELEVATES THE LEVEL OF EXPRESSION AND ACTIVATION OF PROTEIN KINASE D2

*M.Yu. Shabelnik[1], O.V. Kostyuk[2], S.V. Merentsev[3], T.O. Tarasova[4], S.P. Sidorenko[1,] **

[1]*R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, Kiev 03022, Ukraine*
[2]*A.A. Bogomolets National Medical University, Kiev 04053, Ukraine*
[3]*Kiev City Oncological Hospital, Kiev 03115, Ukraine*
[4]*State National Institute of Cancer, Kiev 03022, Ukraine*

Aim: **To test the hypothesis, whether *H. pylori* infection may affect the level of PKD2 expression and/or activation in gastric cancer cells.**
Methods: **Studies were performed on AGS human gastric adenocarcinoma cell line, gastric tissues samples from 36 cases of different histological variants of gastric cancer. Immunohistochemical, cell and molecular biology, bacteriological and biochemical approaches have been used in this study.** ***Results: H. pylori 16S rRNA*** **gene was detected in 97% cases of gastric tumors, and in 83% of cases *cagA* gene was detected. In all tested adenocarcinoma samples *cagA*$^+$ *H. pylori* was revealed. These cases were characterized by high level of PKD1/2 expression and autophosphorylation. In adenogenic cancer samples the presence of *cagA*$^-$ *H. pylori* was identified. Carcinoid and nondifferentiated gastric cancers contain *H. pylori*, with very low numbers of *cagA*$^+$ copies. All cases of gastric tumors with *cagA*$^-$ *H. pylori* had very low levels of PKD1/2 autophosphorylation. AGS cell line infection with *cagA*$^-$ and *cagA*$^+$ *H. pylori* resulted in elevation of PKD2 expression levels in 3.29 and 3.66 times respectively ($p < 0.001$). In cells infected by *cag*$^+$ *H. pylori* the level of PKD2 transphosphorylation was 1.39 higher than in cells infected by *cagA*$^-$ *H. pylori*. For PKD2 autophosphorylation this difference was even higher — 3.27 times ($p < 0.001$).** ***Conclusion: H. pylori*** **infection enhanced the level of protein kinase D2 expression, trans- and autophosphorylation. The level of PKD2 autophosphorylation/activation was higher in AGS cell line inoculated of with *cag*$^+$ *H. pylori* than in AGS cells with *cagA*$^-$ *H. pylori*. These suggest that *H. pylori* induces activation of PKD1/2 and could exploit PKD2 mediated signaling pathways that may contribute to the pathogenesis of gastric cancer.**
Key Words: **gastric cancer, protein kinase D, AGS cell line, *Helicobacter pylori*, CagA.**

Malignant transformation of cells is a complex process, which involves the accumulation of multiple independent mutations that lead to deregulation of cell signaling pathways that control cell growth, proliferation, differentiation and apoptosis [1]. Recent progress in biomedical sciences has identified a number of molecular pathways and cellular mechanisms that underline the multistage process of tumorogenesis and metastasis formation. It was shown that serine/threonine protein kinases could be involved in the integration of different signaling pathways in normal and malignant cells [2].

Protein kinase D (PKD) family within the group of calcium/calmodulin-dependent protein kinases (CAMK) consists of three members: PKD1/PKCμ, PKD2 and PKD3/PKCv. These kinases share high structural homology but differ in cell specific expression, intracellular distribution and, most probably, in substrate specificity and cellular function [2]. Full activation of PKD involves phosphorylation of Ser residues within different structural modules. Transphosphorylation sites in so-called "activation loop" of PKDs are substrate for PKCs phosphorylation. Autophosphorylation sites have been mapped in C-terminus of PKD1 and PKD2 and serves as indicator of kinase activation [3].

It is known that PKDs are activated by a range of cell surface receptors and pharmacological agents that could induce PKDs intracellular redistribution. PKD may be localized in the cytosol and in several intracellular compartments including nucleus, Golgi apparatus, plasma membrane and mitochondria. Depending on site of localization PKDs can be implicated in the regulation of a variety of cellular processes, including Golgi apparatus function and organization, receptors signalling, tumor cell spreading and motility that promote tumor invasion [2, 4].The presence of the nuclear translocation signal peptide may suggest that upon stimulation PKDs are translocated into the nucleus and phosphorylate nuclear targets, such as histone deacetylases and histone chaperone SET [5]. Moreover, indirectly PKDs could be involved in activation of transcription factor NF-κB [6]. In this way, PKD kinases are implicated in epigenetic regulation of gene expression [5].

PKD2 is the main serine/threonine protein kinase of PKD family, which is expressed in AGS cell line. In this cell line PKD2 is activated by gastrin and is a downstream target of PKCs [7, 8]. However, little is known about the expression and activity of PKD2 as well as PKD1 in primary gastric cancer cells.

H. pylori is a widespread chronic infection agent, that is considered to be aetiologycal factor for gastric and duodenal ulcer, MALT-lymphoma and gastric adenocarcinoma [9]. At the same time, the role *H. pylori*

Received: July 20, 2009.
*Correspondence: Fax: 38 (044) 258-16-56
E-mail: svitasyd@yahoo.com

Abbreviation used: AGS – adenocarcinoma gastric cell line; AP-1 – activator protein; CAMK – calcium/calmodulin-dependent protein kinase; Csk – C-terminal Src kinase; MIF – macrophage migration inhibitory factor; NF-kB – nuclear factor -kB; PKC – protein kinase C; PKD – protein kinase D.

infection in pathogenesis of gastric cancer is not completely clarified.

Virulent *H. pylori* strains harbor a *cagA* pathogenicity island for delivery of the bacterial CagA protein into gastric epithelial cells [10, 11]. CagA is transported into the host target cells and subsequently phosphorylated. Clearly this is a mechanism by which *H. pylori* could take control of one or more host cell signal transduction pathways [10]. Presumably the final result of this interaction favors survival of *H. pylori*, irrespective of eventual damage to the host cell. After translocation, CagA is phosphorylated by Src family kinases at the tyrosine residue in the EPIYA sequence repeats [12]. Phosphorylation of CagA is accompanied by high motility and elongation of cells, the so-called hummingbird phenotype [13]. CagA has been shown to interact with a number of host signaling molecules, such as the adaptor molecule Grb-2, tyrosine phosphatase SHP-2, Src, and C-terminal Src kinase (Csk) to induce inactivation of Src kinase and dephosphorylation of cortactin [14]. It was also shown, that infection by *H. pylori* induced signal transduction pathways that involve PKC kinases [15, 16]. PKCs phosphorylate and activate kinases of PKD family [3]. Taking into account that previously we have found heterogeneity in PKD1/2 autophosphorylation in gastric adenocarcinomas [17], in current study we tested the hypothesis whether infection by the *H. pylori* may affect the level of PKD2 expression and/or activation in gastric cancer cells.

MATERIALS AND METHODS

Studies were performed on AGS cell line, gastric tumor samples and surrounding normal tissues of stomach (29 cases of adenocarcinomas with different level of differentiation, 3 — cases of adenogenic cancer, 2 — cases of nondifferentiated cancer, 1 — case of carcinoid tumor, 1 — case of adenoma). Gastric tumors were classified and graded according to WHO classification on the basis of combination of morphologic and clinical characteristics [18]. The usage of tissue samples was approved by the Institutional Review Board and Research Ethics Committee of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, National Academy of Sciences of Ukraine.

Polyclonal rabbit antibodies, that recognize both PKD1 and PKD2 kinases (anti-PKD1/2) and autophosphorylated sites in both PKD1 and PKD2 (anti-pPKD1/2) (kind gift of Prof. VanLint, Belgium), PKD2 (Calbiochem, USA), Ser876 in PKD2 (autophosphorylated pPKD2) (Upstate, USA), Ser 744/748 in PKD2 (transphosphorylated ppPKD2) (Cell Signaling, USA) and goat anti-actin antibodies (Santa Cruz, CA, USA) were used as primary antibodies. EnVision detection system (DAKO, Denmark), goat anti-rabbit IgG-HRP and donkey anti-goat IgG-HRP (Santa Cruz, CA, USA) were applied as a secondary antibodies.

Immunohistochemistry. Tissue samples of all studied cases were fixed in 4% paraformaldehyde and embedded in paraffin. Immunohistochemistry studies of PKD1 and PKD2 were performed on deparaffined tissues sections. We used specific anti-PKD1/2 antibodies that recognized both isoforms. The levels of autophosphorylated PKD1 and PKD2 were used as a criterion of activated PKD1/2 and were accessed with anti-pPKD1/2 serum, which recognize conserved autophosphorylation site in PKD1 and PKD2.

To reduce non-specific background, prior to specific antibodies, sections were treated with normal goat serum and 1% BSA solution. EnVision detection system was used in 45 min second-step incubation. After washing in phosphate-buffered saline peroxidase activity was assayed using DAB. Section were counterstained with hematoxylin for 1 min, embedded in balm, and studied under the microscope.

Isolation and cultivation of H. pylori. For isolation we used samples of pyloric antrum of the stomach from 4 patients with gastric ulcer (Department of Therapy № 2 of A.A. Bogomolets National Medical University, Kiev, Ukraine). For tissue sample transportation we used Stuart's transport medium (Merck, USA). Prior inoculation, material was homogenized in 0.5 ml of sterile PBS or brain heart infusion broth during 1 min. Selective medium Agar pylori (Bio Merieux, France) and nonselective — Columbia agar with 10% of the sheep blood (Bio Merieux, France) were inoculated with gomogenized tissues. Tissues dishes were incubated in GENbox Jar (Bio Merieux, France), in microaerofilic condition that was created by special gas generation packages (Bio Merieux, France) at 37 °C during 7 days.

Typing of bacterial cultures was performed using cultural, morphological, tinctorial and enzymatic criteria. Selected cultures of *H. pylori* were subcultivated on Columbia agar with 10% of horse blood in microaerofilic condition at 37 °C during 48–72 h. For inoculation of AGS cell line we used only "young" cultures of *H. pylori*, that had less than 25% of coccal forms and in size of the 108 CFU on Mac-Farland's scale.

Inoculation AGS cell line with H. pylori. AGS cell line was cultivated with *cagA*$^-$ and *cagA*$^+$ *H. pylori* cultures during 4.5 h and proteins were extracted using Triton-X100 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton-X100, 5 mM EDTA) with cocktail of inhibitors of proteases and phosphatases (Sigma, USA).

Western blot analysis. Western blot analysis was performed using rabbit anti-PKD2, anti-ppPKD1/2 (Ser 744/748), and anti-pPKD2 (Ser876) rabbit sera. The cells from cell cultures were lysed using Triton-X100 lysis buffer with cocktail of inhibitors of proteases and phosphatases (Sigma, USA). Lysates were subjected to SDS-PAGE on 7.5% gels. Proteins were transferred to Immobilon-NC membranes (Millipore, USA) and blocked during overnight incubation with 5% nonfat dried milk in TBS-T. Membranes were incubated with primary antisera at 4 °C for 12 h and with secondary antibodies for 2 h. For semiquantitative evaluation of Western blot analysis Total lab software version 2.01 (Phoretix, Nonlinear Dynamics Ltd) was applied.

DNA isolation and polymerase chain reaction for H. pylori identification. DNA isolation was per-

formed using TRI reagent (Sigma, USA) according to manufacturers protocol. DNA pellet was dissolved in TE buffer. For polymerase chain reaction (PCR) we used 0.5 μg of DNA for each probe. The universal primers for *H. pylori* detection in gastric tissue included two oligonucleotides designed to amplify *16S rRNA* (Maxim Biotech. Inc., USA), (GenBank Acession No.: U00679) the 5'Oligo: CTGGAGAGACTAAGCCCTCC and the 3'Oligo: ATTACTGACGCTGATTGTGC, which generated a 110-bp product. PCR amplification with specific primers was performed according to standard protocol: thirty five cycles, with each cycle consisting of denaturation step at 94 °C, annealing step at 58 °C and extension step at 72 °C. The specific primers for PCR amplification of *cagA H. pylori* (Helicopol Ca, Russia), which generated a 404-bp product, were used for identification of *cagA*$^+$ strains of *H. pylori*. Thirty five cycles were performed, with each cycle consisting of denaturizing step at 94 °C, annealing step at 52 °C, and extension step at 72 °C. Each experiment included negative and positive controls provided by manufacturers.

Statistical analysis. Statistical significance of differences was evaluated by Student's *t*-test.

RESULTS AND DISCUSSION

Starting from our previous results that showed heterogeneity of primary adenocarcinoma cancer samples by expression levels of PKD1 and PKD2 and autophosphorylation/activation, we found that different histological variants of gastric cancer also vary in the level of PKD1/2 autophosphorylation. The high level of PKD1/2 autophosphorylation was detected in adenocarcinomas with moderate level of differentiation (Fig. 1, *a*), whereas in adenogenic cancer the level of PKD1/2 autophosphorylation was very low or undetectable (Fig. 1, *b*). At the same time, benign tumor (adenoma) demonstrated high level of PKD1/2 autophosphorylation (Fig. 1, *c*). Heterogeneity of gastric cancer primary samples by PKD1/2 autophosphorylation could depend not only on the level of cell differentiation [17], but also reflect tumor cell activation with infectious agents. Since *H. pylori* infection was shown to trigger PKC-mediated signaling pathway [15, 16], and activity (autophosphorylation) of PKD kinases is regulated by PKCs [2, 19], we analyzed the presence of *H. pylori* in the primary gastric tumor samples and addressed the question whether *H. pylori* could induce PKD phosphorylation.

The *16S rRNA* gene of *H. pylori* is a highly specific target for amplification and has been used previously to help reclassify these bacteria [20]. Weiss et al. [21] have demonstrated the specificity of unique *H. pylori 16S rRNA* gene primers to identify the bacteria in paraffin-embedded gastric biopsy specimens. In primary tissue samples we found that 97% (35 from 36) cases of examined gastric tumors contain *H. pylori 16S rRNA* (Fig. 2, *a*) that is comparable to the previously published data [22].

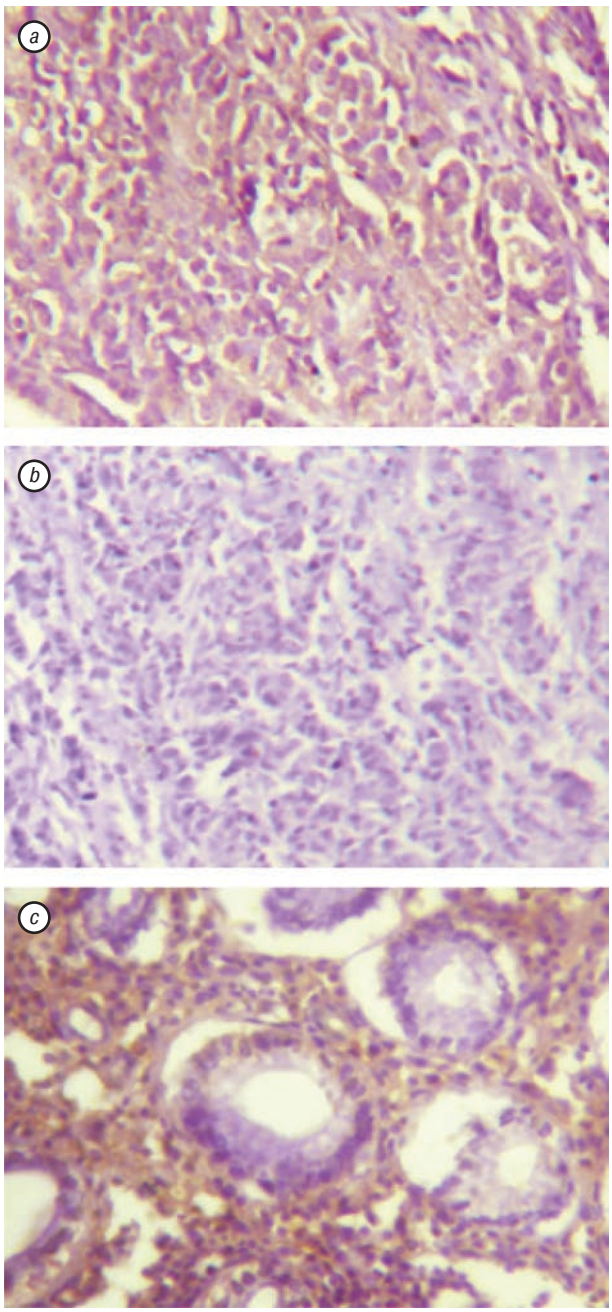

Fig. 1. The level of PKD1/2 autophosphorylation in gastric tumors, immunohistochemical staining. *a*, adenocarcinoma; *b*, adenogenic cancer; *c*, adenoma (X400)

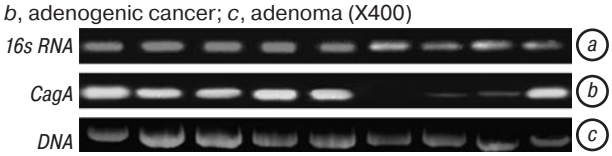

Fig. 2. PCR analysis of DNA isolated from gastric tissue samples. The presense of 16S rRNA (*a*) of *H. pylori* identify the bacteria from frozen (1–4, 7) and in paraffin-embedded gastric biopsy specimens (5–6, 8). *b*, *cagA*$^+$ from *H. pylori*. *c*, Control of assayed material (DNA). 1–5 — adenocarcinomas, 6 — adenogenic cancer, 7 — carcinoid cancer, 8 — nondifferentiated gastric cancer, 9 — positive control

Some strains of *H. pylori* have been shown to be more pathogenic than others. Strains that possess CagA are associated with increased severity of gastritis and with additional risk for developing atrophic gastritis and gastric cancer [23]. Moreover, it has been demon-

strated that after *H. pylori* infection, CagA is translocated into the epithelial cells, and it undergoes tyrosine phosphorylation in the host cells. The phosphorylated form of CagA might function as a phosphatase that regulates host cell growth [24]. As we can find out, *cagA* gene was detected in 83% (30 from 36) cases of primary tumor tissue samples (Fig. 2, *b*). It should be noted that *H. pylori cagA*$^+$ was revealed in all tested 29 adenocarcinoma samples as well as gastric adenoma tissue sample (see Fig. 2, *a*, *b*, tracks 1–5; other data not shown). In two samples of adenogenic cancer *cagA*$^-$ gene *H. pylori* was identified, however one case of adenogenic cancer can be considered as *H. pylori* negative, since *H. pylori 16S rRNA* gene was not detected here (see Fig. 2, *a*, *b*, track 6 and data not shown). In carcinoid and nondifferentiated gastric cancer we found the presence of *H. pylori*, with very low numbers of *cagA* copies (see Fig.2 *a*, *b*, tracks 7 and 8).

Since in our studies the presence of *cagA*$^+$ *H. pylori* in tumor tissues was correlated with the moderate to high level of PKD1/2 autophosphorylation, we addressed the question whether *H. pylori* infection may affect the level of PKD2 expression and/or trans- and autophosphorylation. To test this hypothesis we used the model gastric cancer cell line AGS which was infected with *H. pylori*. Cultivation of AGS cell line with *cagA*$^-$ or *cagA*$^+$ *H. pylori* cultures during 4.5 h caused the elevation of PKD2 expression level in comparison with the control cultures, moreover, it resulted in PKD2 transphosphorylation (ppPKD2) and autophosphorylation (pPKD2) (Fig. 3).

Control cell culture did not demonstrate PKD2 transphosphorylation that is PKC-dependent. PKD2 in these cultures was not activated and, accordingly, was not autophosphorylated (see Fig. 3, *a*). At the same time, inoculation of AGS cell line with *H. pylori* led to substantial PKD2 trans- and autophosphorylation reflecting the activation of this kinase. Furthermore, the level of PKD2 autophosphorylation in AGS cell line inoculated with *cagA*$^+$ *H. pylori* was much higher than that in cells inoculated with *cagA*$^-$ *H. pylori* (see Fig. 3, *a*). To evaluate *H. pylori* induced changes in PKD2 expression and phosphorylation we have performed densitometric analysis of Western blots with normalization to the level of actin expression (see Fig. 3, *b*). As it is shown on the left panel of Fig. 3, *b*, infection with *cagA*$^-$ and *cagA*$^+$ of *H. pylori* led to elevation of PKD2 expression by 3.29 and 3.66 times respectively ($p < 0.001$). *H. pylori* infection dramatically increased both trans- and autophosphorylation of PKD2. It worth to note that in cultures with *cagA*$^+$ *H. pylori* the level of transphosphorylation (PKC-dependent) was by 1.39 times higher than in cultures infected with *cagA*$^-$ *H. pylori* ($p < 0.001$). For PKD2 autophosphorylation this difference was even higher — 3.27 times ($p < 0.001$) (see Fig. 3, middle and right panel).

The development of cancer is often associated with chronic inflammation [25]. Inflammation is linked to all stages of tumor development — initiation, progression and metastasis. Although the connection between chronic inflammation and cancerogenesis has been well established, the underlying mechanisms remain unclear [25]. Recent studies have begun to unravel signaling pathways linking inflammation and cancer [26].

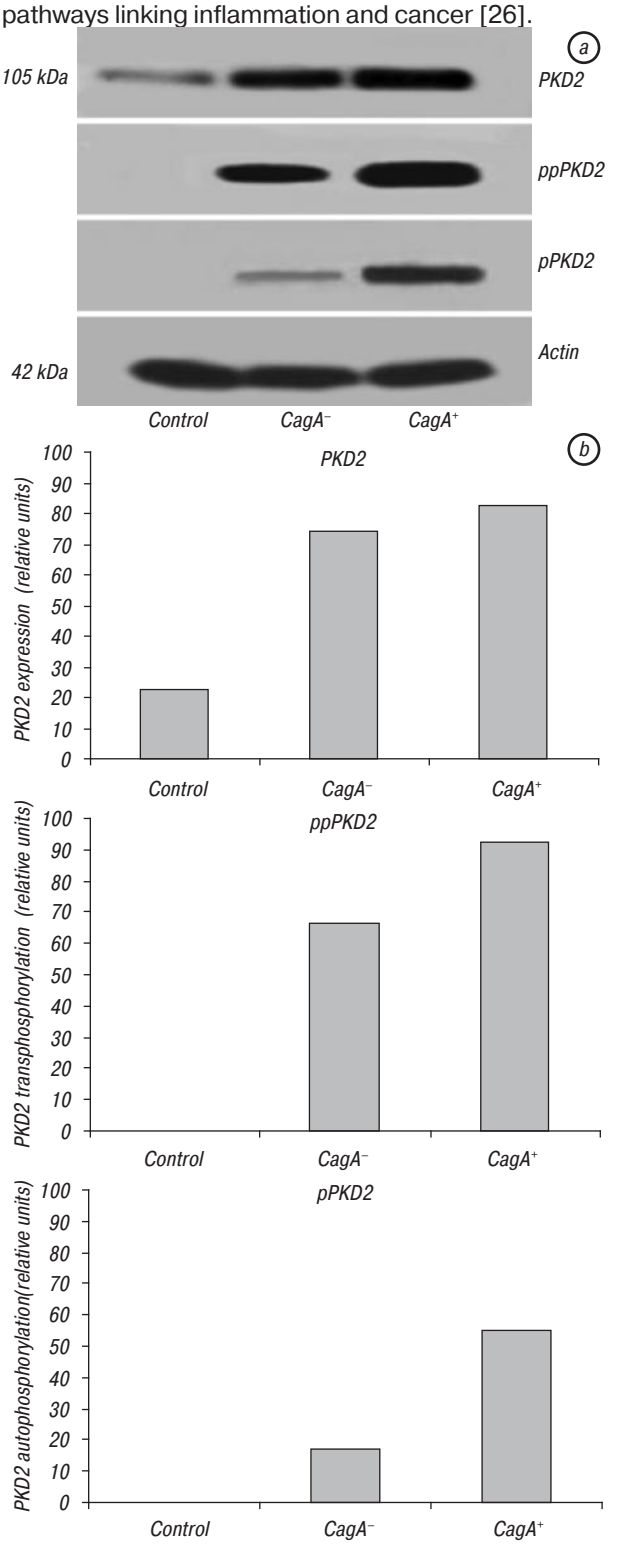

Fig. 3. Protein expression, trans- and autophosphorylation of PKD2 in AGS cell line after *H. pylori* infection. *a*, Western blot analysis of PKD2 expression, transphosphorylation (ppPKD1/2), and autophosphorylation (pPKD2). The level of actin expression served as loading control. Control — noninfected AGS cell line, *cagA*$^-$ — AGS cell line infected with *cagA*$^-$ culture of *H. pylori*. *cagA*$^+$ — AGS cell line infected with *cagA*$^+$ culture of *H. pylori*. *b*, Densitometry of Western blot analysis with normalization to actin ($p < 0.001$)

H. pylori that infects over half of the world's population, usually persists in the gastric mucosa [27]

and confers risk of serious diseases, including peptic ulceration and gastric neoplasia [28]. *H. pylori* is the first bacterium to be classified as a definite carcinogen because of its epidemiologic relationship to gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue MALT lymphoma. Understanding how this bacteria interacts with its host and affects biological functions of epithelial cells via interaction with different intracellular signaling pathways is essential for designing the strategy for optimized therapy [29].

Recently the mechanisms *H. pylori* interaction with host cells have been clarified. *H. pylori* possesses over 30 genes related to the expression of outer membrane proteins. Several of these proteins have been classified as adhesins, suggesting multiple and perhaps redundant or variable modes of attachment to cell surface. The best studied *H. pylori* adhesins are outer membrane proteins that bind carbohydrate moieties in host cell glycoproteins [29]. Bacterium also use CD74 for adhesion to epithelial cells [30].

There are known several distal outcomes of *H. pylori* interaction with gastric epithelial cell for example, activation of transcription factors NF-κB and AP-1, induction of secretion of MIF, IL-8, IL-1β [30, 31], but the mechanisms of signal transduction and intracellular pathways utilized by this bacterium was not explored.

In our studies we tested the hypothesis whether *cagA*$^+$ and *cagA*$^-$ *H. pylori* can affect the PKD2 expression and activation.

Our study revealed the presence of *H. pylori* in all examined 29 adenocarcinoma samples that are characterized by high and moderate level of PKD1/2 autophosphorylation. Moreover, all these cases also were *cagA*$^+$. Adenogenic and nondifferentiated gastric cancer, and also carcinoid ones had very low level of PKD1/2 aurophosphorylation and are *cagA*$^-$ or carry low numbers of *cagA*. At the same time, benign tumor (adenoma) demonstrated high level of PKD1/2 autophosphorylation and the presence of *cagA*$^+$ *H. pylori*. Heterogeneity of primary gastric cancer samples in PKD1/2 autophosphorylation could depend on the level of cell differentiation, but also reflect tumor cell activation with infectious agents. Althoug the percent of *H. pylori* positive gastric samples vary depending on age, geographical localization and ethnicity, *H. pylori* was detected in vast majority of adenocarcinoma samples [27, 32]. According to our data, *H. pylori* was detected in all tested adenocarcinoma samples, moreover, all studied adenocarcinoma cases were *cagA*$^+$.

Correlation between the presence of *cagA* positive *H. pylori* and PKD1/2 phosphorylation may point on causal link between infection and PKDs activation. To test this assumption we employed experimental model system. Indeed, *H. pylori* infection of AGS cell line induced autophosphorylation/activation of PKD2 (see Fig. 3). This activation was PKC-dependent, since PKD2 was phosphorylated at site Ser 744/748, which is the target of PKC (see Fig. 3). Moreover, we found that infection with either *cagA*$^-$ and *cagA*$^+$ *H. pylori* also elevated the level of PKD2 expression in AGS cell line. Markedly, the *cagA*$^+$ *H. pylori* was more potent than *cagA*$^-$ *H. pylori* in elevation of PKD2 level (see Fig. 3, *a*). It is obvious that increase of PKD2 activity was not dependent on the level of PKD2 expression since in control cultures PKD phosphorylation was not detected.

There are several lines of evidence of the functional link between *H. pylori* infection and PKDs activation. First, *H. pylori* infection was shown to trigger PKC-mediated signaling pathway [15, 16], and PKCs regulate activity (autophosphorylation) of PKD kinases [2, 19]. Second, both *H. pylori* and PKDs are involved in NF-κB activation [27, 30]. Third, CagA targets an important cellular E-cadherin/β-catenin pathway, which regulates epithelial junction formation, epithelial cell adhesion, and control cell growth [33]. PKDs activity is also involved in regulation of E-cadherin/β-catenin pathway especially in tumor cells [34]. Fourth, both PKDs and *H. pylori* are linked to regulation of HDAC (histon deacetylases), which are involved in epigenetic regulation of gene expression [28, 35]. Fifth, both regulate matrix metalloproteinase expression [36–38].

All these suggest that in order to affect host cell biological programs, *H. pylori* may use PKDs-mediated signalling pathways.

In conclusion, using the model system of gastric cancer cell line AGS we have shown that *H. pylori* infection enhanced the levels of expression, trans- and autophosphorylation of protein kinase PKD2, that may lead to activation of signal transduction pathways, promoting cell proliferation and tumor invasion. We also found that the level of PKD2 autophosphorylation/activation was higher in AGS cell line inoculated of with *cagA*$^+$ *H. pylori* than that in AGS cells with *cagA*$^-$ *H. pylori*. *H. pylori* induced activation of PKD1/2 could contribute to the pathogenesis of gastric cancer.

ACKNOWLEDGEMENTS

This work was supported in part by Ukrainian governmental grants 010U000784, 0105U005557 and 0107U002244.

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