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Urokinase plasminogen activator receptor is expressed in invasive cells in gastric carcinomas from high- and low-risk countries

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Gastric cancer is the second cancer causing death worldwide. Both incidence and mortality rates vary according to geographical regions. The receptor for urokinase plasminogen activator (uPAR) is involved in extracellular matrix degradation by mediating cell surface associated plasminogen activation, and its presence on gastric cancer cells is linked to micrometastasis and poor prognosis. Immunohistochemical analyses of a set of 44 gastric cancer lesions from Costa Rica showed expression of uPAR in cancer cells in both intestinal subtype (14 of 27) and diffuse subtype (10 of 17). We compared the expression pattern of uPAR in gastric cancers from a high-risk country (Costa Rica) with a low-risk country (Norway). We found uPAR on gastric cancer cells in 24 of 44 cases (54%) from Costa Rica and in 13 of 23 cases (56%) from Norway. uPAR was seen in macrophages and neutrophils in all cases. We also examined the nonneoplastic mucosa and found that uPAR was more frequently seen in epithelial cells located at the luminal edge of the crypts in cases with *Helicobacter pylori* infection than in similar epithelial cells in noninfected mucosa ($p = 0.033$; $\chi^2 = 4.54$). In conclusion, the expression of uPAR in cancer cells in more than half of the gastric cancer cases suggests that their uPAR-positivity do not contribute to explain the different mortality rates between the 2 countries, however, the actual prevalence of uPAR-positive cancer cells in the gastric cancers may still provide prognostic information.

Key words: uPAR, gastric cancer, *Helicobacter pylori*, gastritis, immunohistochemistry
Abbreviations: CK: cytokeratin; ECM: extracellular matrix; mAb: monoclonal antibodies; pAbs: polyclonal antibodies; TNP: trinitrophenyl; uPA: urokinase-type plasminogen activator; uPAR: uPA receptor
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Gastric cancer is the second most common cancer causing death worldwide after lung cancer and is a final result of the stepwise process initiated by environmental factors including diet and *Helicobacter pylori* infection.[1–3] In particular, *H. pylori* infection is one of the most recognized risk factors for this malignancy.[4–6] Both the incidence and mortality rates of gastric cancer present substantial variations according to geographical regions (between and within countries).[1] The factors explaining these variations remain unknown so far given the complexity and multifactorial nature of the disease. Costa Rica is one of the countries with highest incidence and mortality rates for gastric cancer worldwide. In contrast, most of western European countries present low incidence and low mortality rates for this malignancy.[1,7]

Among the key events for cancer development and progression are neoplastic cell invasion into the adjacent normal tissue and eventually metastasis. Invasion and metastasis are facilitated by a number of proteinases capable of degrading the extracellular matrix (ECM).[8,9] Plasmin can degrade major ECM proteins like fibrin, fibronectin and laminin and in

addition can activate other matrix degrading proteinases. Plasmin is formed on cell surfaces after proteolytic cleavage of its zymogen plaminogen by urokinase-type plasminogen activator (uPA).[10] uPA mediates plasminogen activation after binding with high affinity to its specific receptor (uPAR), a 3-domain GPI-linked cell surface protein.[11] uPAR is predominantly seen on inflammatory cells and to a lesser extent on cancer cells in areas of cancer invasion in several types of cancer including gastric, colon, breast and oral cancer.[12–17]

In gastric cancer lesions, uPAR expression has been observed in macrophages, endothelial cells and cancer cells located at the invasive front of the tumors.[15,18,19] Increased expression of uPAR antigen or mRNA in tissue extracts and blood from patients with gastric cancer have been associated with some clinico-pathological aspects of the disease including poor prognosis.[18–26] Interestingly, studies in bone marrow aspirates from curatively resected patients with gastric cancer show that those cases with uPAR-positive cancer cells disseminated into the bone marrow have worse prognosis than patients with disseminated uPAR-negative cancer cells.[27–29] A significant association between high expression levels of uPAR in primary tumors and uPAR-positive disseminated tumor cells was also reported.[30] These observations suggest that elevated expression of uPAR in tumors and particularly in cancer cells may be an indication of more aggressive gastric cancers. Therefore, uPAR expression in cancer cells may represent an important prognostic marker for patients with gastric cancer. In addition, studies in gastric cancer cell lines have reported increased uPAR mRNA levels when neoplastic cells are cocultured with *H. pylori*,[31,32] suggesting that *H. pylori* may induce the expression of uPAR in gastric mucosa. This may link uPAR with early steps of gastric carcinogenesis, which contributes to explain the association between *H. pylori* and gastric cancer and the correlation observed between the incidence rates of this malignancy and the prevalence of the bacterium.[33]

This study has been conducted to compare the expression and presence of uPAR in gastric cancer cells in cases from high- and low-risk countries (Costa Rica and Norway, respectively) and to explore the possible connection between uPAR expression in gastric mucosa and *H. pylori* infection.

Material and Methods

Tissue samples

Formalin-fixed and paraffin-embedded gastric cancer tissue (neoplastic and nonneoplastic) were obtained postoperatively from 44 gastric cancer cases in 2 hospitals in Costa Rica (Max Peralta Hospital and Rafael Angel Calderón Guardia Hospital) and 23 cases from Bergen, Norway (Haukeland University Hospital). Histopathological information was collected from all the 67 cases. This histopathological classification was given according to the Laurén Classification System (Norwegian cases) and Japanese Classification System (Costa Rican cases). For the purposes of this study, Costa Rican cases were reclassified according to Laurén Classification System following established criteria given by the Japanese Gastric Cancer Association.[34] Thus, of the 44 Costa Rican cases, 27 were classified as intestinal subtype and 17 as diffuse subtype. Among the 23 Norwegian cases, 15 were intestinal subtype and 8 were diffuse subtype. According to the International Union Against Cancer (IUCC) criteria, the 44 Costa Rican cases were staged as follows: 16 (36%) stage IA, 3 (7%) stage IB, 5 (11%) stage II, 3 (7%) stage IIIA, 4 (9%) stage IIIB, 7 (16%) stage IV and in 6 (14%) cases it was not known (pN was missing on the records). Accordingly, among the 23 Norwegian cases, 0 had stage IA, 1 (4%) stage IB, 6 (26%) stage II, 0 stage IIIA, 1 (4%) stage IIIB and 15 (66%) stage IV. Survival data could not be obtained from the Costa Rican cases, but they were available for the 23 Norwegian cases. The study was approved by the respective Ethical and Research Committees of each institution (Costa Rica: VI 742-94-571, VI 742-99-340; Norway: REK 053228) and performed in accordance with the World Medical Association Declaration of Helsinki, 1996.

Antibodies

Affinity purified monoclonal antibodies (clones R2 and R4) and rabbit polyclonal antibodies (pAbs) against human uPAR have been described previously.[35,36] mAbs against cytokeratin-19 (clone RCK108), cytokeratins (clones AE1/AE3), CD68 (clone KP1), rabbit pAbs against *H. pylori* (code no. B0471), FITC-conjugated goat anti-mouse IgG and nonimmune rabbit IgG were purchased from Dako (Glostrup, Denmark). FITC-conjugated goat anti-rabbit IgG, Cy3-conjugated goat anti-mouse and Cy3-conjugated goat anti-rabbit were obtained from Jackson Immunoresearch (West Grove, PA). Monoclonal antibody directed against trinitrophenyl hapten (TNP, IgG1) was previously described.[37]

Immunoperoxidase staining

Three to four micrometer paraffin-embedded tissue sections were deparaffinized with xylene and hydrated in gradual series of ethanol-water dilutions. For uPAR R2, uPAR pAb, both cytokeratins (CKs), anti-*H. pylori* and anti-TNP, sections were pretreated with Proteinase-K (10 μg/mL) for 25 min at 37°C. For R4 immunohistochemistry, sections were heat-treated in a T/T Micromed microwave processor (Milestone, Sorisol, Italy) at 98°C for 15 min in target retrieval solution (code no. S1699 pH 6.0; Dako). Endogenous peroxidase activity was blocked by incubation in 1% hydrogen peroxide solution for 15 min. The primary antibodies were diluted in antibody diluent (Dako) and incubated for 2 hr in Shandon racks (Thermo Shandon, Pittsburg, PA) at the following dilutions: uPAR R2 0.9 μg/mL, uPAR R4 5.1 μg/mL, uPAR pAb 1.8 μg/mL, CKs 1:300, anti-*H. pylori* 1:150. Subsequently, the primary antibodies were detected with EnVision reagent, either anti-mouse IgG or anti-rabbit IgG horseradish peroxidase-conjugated polymers (Dako). The reactions were visualized by incubating the sections with NovaRED (Vector

Laboratories, Burlingame, CA) or DAB Chromogen (Dako) (for *H. pylori* pAbs) according to manufacturer's instructions and counterstained with Mayer's haematoxylin.

Negative controls

The sections were pretreated in the same way as previously described for all the antibodies. The polyclonal anti-uPAR was substituted with normal rabbit immunoglobulin (code No. X903 Dako) incubated in an Ig concentration equivalent to that used for the specific primary antibody. Monoclonal antibodies R2 and R4 were substituted with anti-TNP mAb incubated at the same concentrations as those for R2 and R4.

Immunofluorescence staining

For the double immunofluorescence analyses, sections were initially processed as mentioned earlier for uPAR pAbs, using proteinase-K pre-treatment. uPAR pAbs (2.4 μg/mL) were diluted in Dako antibody diluent and incubated 2 hr at room temperature, together with a mixture of mouse monoclonal antibodies against CK-19 and CK AE1/AE3 (CKs, 1:300). The uPAR pAb was detected with Cy3-conjugated goat anti-rabbit IgG, 1:200 and the mixture of monoclonal antibodies against CKs with FITC-conjugated goat anti-mouse IgG, 1:200. To carry out double staining for uPAR and *H. pylori*, the sections were heat-treated by using target retrieval solution (code no. S1699 pH 6.0; Dako). The monoclonal anti-uPAR R4 (5.1 μg/mL) was diluted in antibody diluent (Dako) together with anti-*H. Pylori* pAbs (1:150) and incubated on the tissue sections for 2 hr at room temperature. The antibodies were subsequently detected with Cy3-conjugated goat-antimouse IgG, 1:200 and FITC-conjugated goat anti-rabbit IgG, 1:200, respectively. After brief rinses with TBS, the sections were mounted with Prolong Gold antifade (Molecular Probes, Eugene, OR).

For the triple immunofluorescence analyses, Zenon antibody-labelling technology (Molecular Probes, Eugene, OR) was applied, following the manufacturer's instructions. Briefly, sections were heat-treated with target retrieval solution (code no. S1699 pH 6.0; Dako) as described mentioned. Anti-CD68 (clone KP1 and IgG1) was linked to Zenon 647 IgG1 according to the manufacturer's instructions, complexed anti-CD68 and mixed with uPAR pAbs (2.4 μg/mL) diluted in antibody diluent (Dako). The mix of the 2 antibodies was added to the sections and incubated 2 hr at room temperature. After washing with TBS, uPAR pAbs were detected with Cy3-conjugated goat anti-rabbit (1:200). Subsequently, CKs (clones AE1/AE3) was linked to Zenon 488 IgG1 according to the manufacturer's instructions and incubated on the tissue sections overnight at 4°C. Finally, tissue sections were washed with TBS and mounted with Prolong Gold antifade (Molecular Probes, Eugene, OR).

Confocal Microscopy

The double and triple stained sections were analyzed using a confocal laser-scanning microscope, LSM 510 META (Carl Zeiss, Jena, Germany) equipped with a 488 nm Argon laser, a 543 nm HeNe1 laser and a 633nm HeNe2 laser. The images were obtained using the lambda mode (pinhole diameter 135 μm) collecting fluorescent signals from 509 to 595 nm wavelength (double staining) or 509–691 nm wavelength (triple staining). For separation of the specific fluorescence signals, we first obtained FITC, Cy3, Alexa fluor 647 and erythrocyte autofluorescence emission spectra from single-fluorophore stained sections. From double- and triple-labeled sections, the collected fluorescence signal was separated by the emission fingerprinting using the above emission spectra to separate the individual fluorescence signals as described.[38] Nomarsky differential interference contrast (phase image technique) was used to show the tissue structures revealed by refractive index inhomogeneities.

Scoring for the immunoperoxidase stainings

The sections stained for R2 mAb and uPAR pAbs were evaluated by 2 independent investigators. uPAR immunoreactivity in cancer cells was scored based on the estimated percentage of positive cells seen in the whole section. Thus, the percentage of positive cancer cells were grouped into the following categories: 0, (no uPAR positive cancer cells detected); 1, less than 1% positively stained cancer cells (some few observed at the invasive edges of the tumor); 2, between 1 and 5% positive cancer cells; 3, between 5 and 10% positive cancer cells and 4, more than 10% positively stained cancer cells (Fig. 2).

H. pylori immunoreactivity was scored based on the density of bacteria and the number of crypts containing bacteria into the following categories: −, no evidence of *H. pylori* on the section; +, less than 3 crypts with small clusters of bacteria; ++, either less than 3 crypts with dense clusters of bacteria or more than 3 crypts with small clusters and +++, 3 or more crypts with dense clusters of bacteria (Figs. 4*h* and 4*i*).

To assess the relation between uPAR expression in epithelial cells and *H. pylori* infection, tissue sections from adjacent normal nonneoplastic gastric mucosa stained for R2 mAb were evaluated independently by 2 investigators. This evaluation was blinded regarding the *H. pylori* status and was based on the estimated percentage of crypts showing uPAR-positive epithelial cells (Fig. 4*c*) as follows:0, 0–5% positive crypts; 1+, 5–30% positive crypts; 2+, 30–60% positive crypts and 3+, more than 60% positive crypts.

Statistical analysis

χ^2 analysis was performed to evaluate the possible differences regarding: the frequency of cases having uPAR-positive cancer cells between Costa Rican and Norwegian cases, the frequency of cases having uPAR-positive cancer cells in intestinal versus diffuse subtypes, the *H. pylori* positivity between the 2 countries and the association between uPAR expression in gastric epithelial cells and *H. pylori* infection. The association between uPAR immunoreactivity in cancer

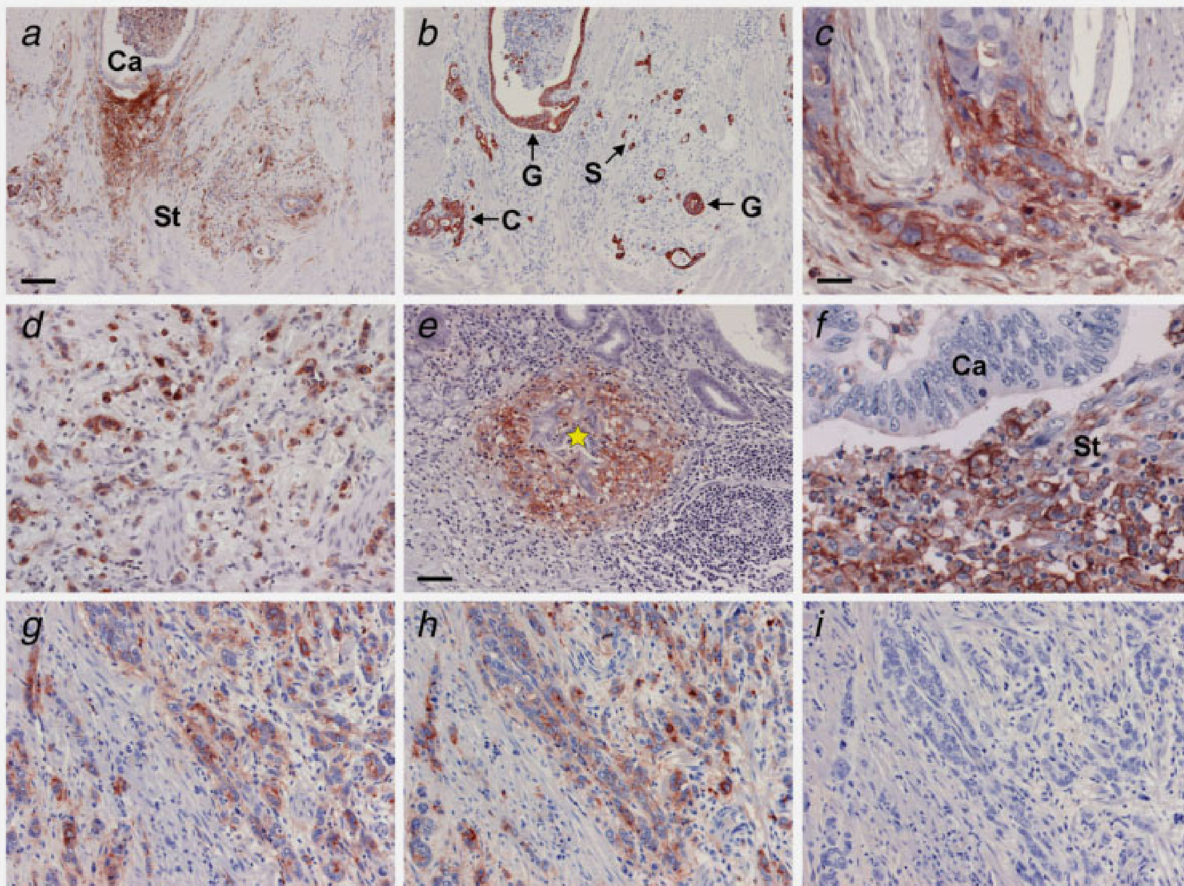

Figure 1. Immunoperoxidase staining for uPAR in gastric cancer. Paraffin sections from intestinal subtype (*a–c, e–f*) and invasive diffuse subtype (*d, g–i*) were incubated with pAbs against uPAR(*a, c, g*), R2 mAb against uPAR (*d–f, h*), mAb against CK (*b*) and a mAb against TNP (*i*). *a–b* and *g–i* represent adjacent sections. In (*b*), CK-positive neoplastic single cells (S), cell clusters (C) and glands (G) in an intestinal subtype gastric cancer case are shown. In the gastric cancer of intestinal subtype, uPAR is focally upregulated in the stromal tissue at the invasive front (*a*; St: stroma, Ca: cancer, *c*), while in the diffuse subtype the uPAR-positive cells are widespread within the tumor (*d*). The intramucosal carcinoma with incipient gastric cancer cells (indicated by a star in *e*) is accompanied by uPAR expression in the adjacent stroma. Macrophages and neutrophils located in the stromal area connected to the invasive front are the main producers of uPAR in gastric cancer (*f*; St: stroma, Ca: cancer). An identical staining pattern is seen with pAb (*g*) and R2 mAb (*h*) against uPAR, while no specific staining is seen with the mAb against TNP (*i*). Sections were counterstained with haematoxylin. Scale bars: ~100 μm (*a–b*), ~25 μm (*c–d, f*), ~50 μm (*e, g–i*).

cells and UICC stage was assessed by χ^2 statistics of exact probabilities. $p = 0.05$ was considered statistically significant in all cases.

Results

uPAR expression in intestinal and diffuse subtypes of gastric cancer

Immunoperoxidase staining for uPAR in both intestinal and diffuse histological subtypes revealed uPAR expression in all the 44 cases (Costa Rica). In gastric cancers of intestinal subtype, uPAR was mainly seen in the stroma along the invasive front of the tumors (Figs. 1*a* and 1*c*) with the most intense signal being observed in areas with abundant invasion of neoplastic single cells (Figs. 1*a* and 1*b*). In contrast, in gastric cancer of diffuse subtype the uPAR-positive cells were seen both in the invasive front and in central areas of the tumors as single cancer cells and/or small clusters of cancer cells (Fig. 1*d*). The expression of uPAR in various cell types along the invasive front in both intestinal and diffuse subtype was histologically complex, but at the general level the uPAR-positive cells included cancer cells, macrophages and neutrophils (Figs. 1*a,* 1*c,* and 1*f*). In some cases, positive fibroblasts and nerve bundles were also observed (not shown). In 7 cases with intramucosal carcinoma, all of them intestinal subtype, uPAR staining was seen in the adjacent stroma, suggesting that uPAR is upregulated early during gastric cancer development (Fig. 1*e*).

The specificity of the uPAR immunoreactivity was based on the analysis of a series of positive and negative controls. An identical uPAR staining pattern was obtained with 3

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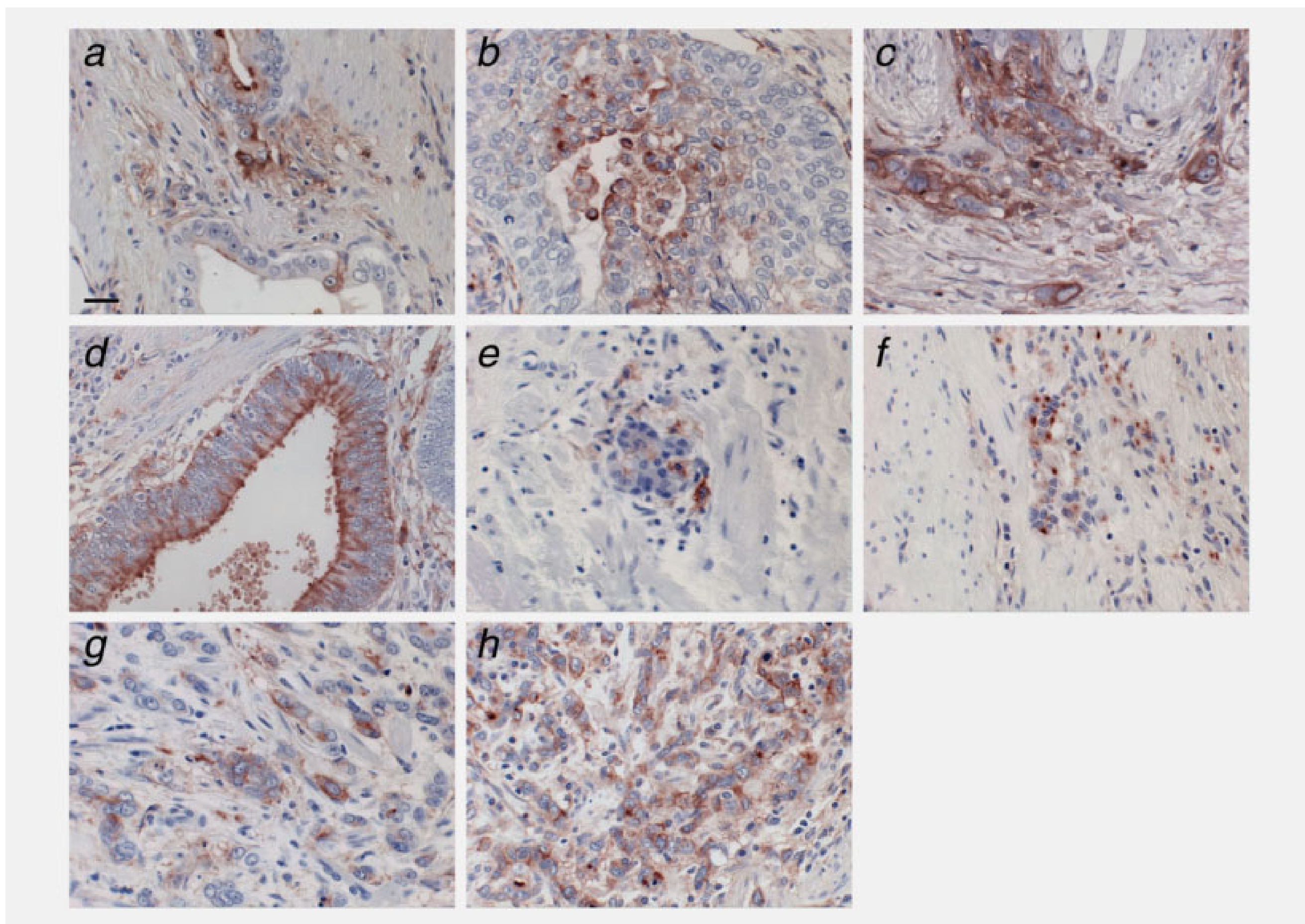

Figure 2. uPAR immunoperoxidase staining for semiquantitative assessment of uPAR-positive gastric cancer cells. Paraffin sections from intestinal subtype (*a–d*) and diffuse subtype (*e–h*) gastric carcinomas were processed for immunoperoxidase staining with pAbs against uPAR and semiquantitatively evaluated for their presence of uPAR-positive cancer cells (see Material and methods). Examples of cases scored as <1% uPAR-positive cancer cells (*a, e*), 1–5% uPAR-positive cancer cells (*b, f*), 5–10% uPAR-positive cancer cells (*c, g*), >10% uPAR-positive cancer cells (*d, h*). Scale bar: ~25 μm (*a–h*).

different anti-uPAR antibodies (R2, R4 and pAbs) in 28 gastric cancer cases (Figs. 1*g* and 1*h*). As negative controls, we substituted R2 and R4 uPAR mAbs with a mAb against TNP and the uPAR pAbs with normal rabbit immunoglobulin. No specific staining was obtained with these 2 antibodies when incubated at similar immunoglobulin concentrations as the respective anti-uPAR antibody preparations (Fig. 1*i*).

uPAR is expressed in gastric cancer cells in both intestinal and diffuse subtypes

Our immunoperoxidase staining suggested that uPAR-positive cancer cells are present in both intestinal (Figs. 2*a*–2*d*) and diffuse subtype of gastric cancer (Figs. 2*e*–2*i*). To further substantiate this observation, immunoflourescence analyses for uPAR in combination with CKs and/or CD68 were performed in a subset of 9 cases, from both histological subtypes, with particularly complex uPAR expression pattern. In intestinal subtype, uPAR was present in both CK-positive cancer cells and in adjacent CD68-positive macrophages (Figs. 3*a*–3*c*), whereas uPAR in diffuse subtype was predominantly seen in CK-positive cancer cells (Figs. 3*d*–3*f*).

uPAR is expressed by cancer cells in both Costa Rican and Norwegian gastric cancers

Based on our uPAR immunoperoxidase staining, we first evaluated the frequency of uPAR-positive cancer cells in all the 44 gastric cancer cases from Costa Rica. In 24 of the 44 cases (54%) uPAR-positive cancer cells were observed, with a similar frequency in intestinal and diffuse subtypes ($p = 0.65$; $\chi^2 = 0.21$). No association between uPAR immunoreactivity in cancer cells and tumor stage was demonstrated ($p = 0.54$; Table 1). Because gastric cancer incidence and mortality rates markedly vary depending on geographical location,[7] we then compiled a series of 23 cases from the low risk country (Norway) with a similar distribution of intestinal and diffuse subtype cases as compared to the Costa Rican series. We tested whether the frequency of uPAR-positive cancer cells was similar to that in the Costa Rican material (Table 1). In general,

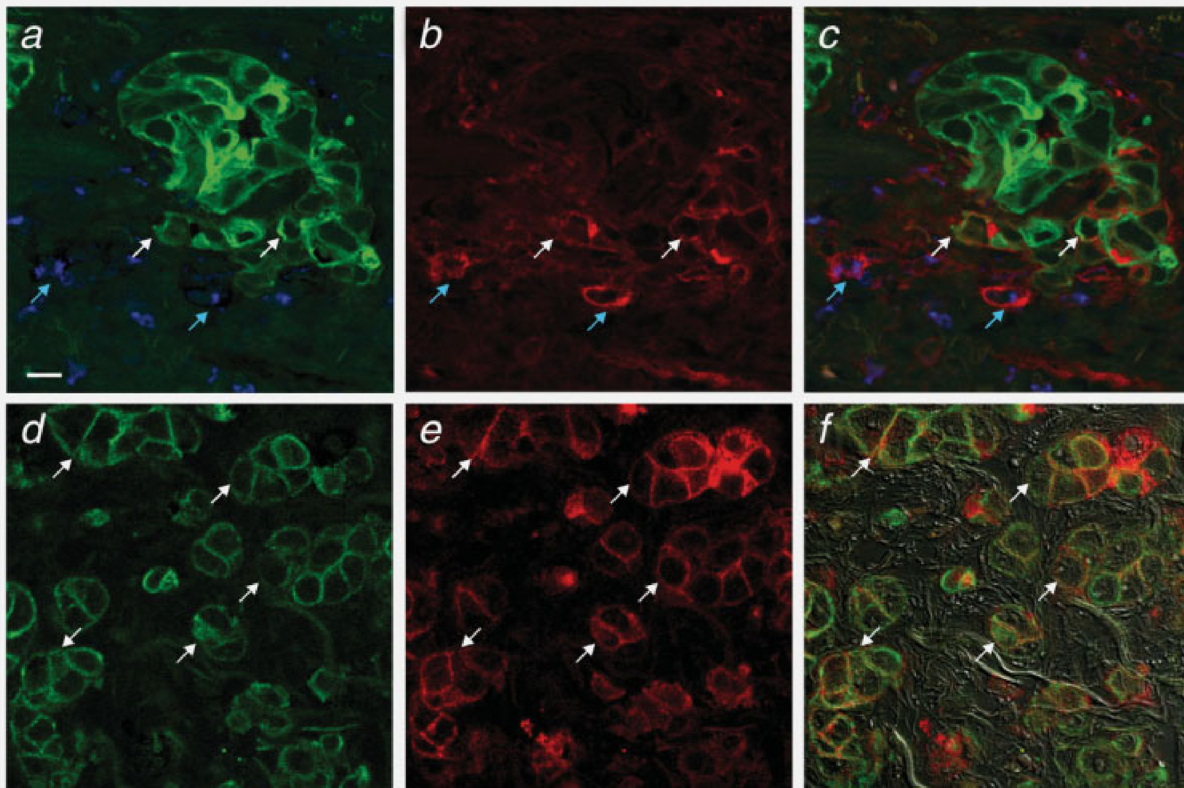

Figure 3. Immunofluorescence analyses for identification of uPAR in cancer cells in gastric cancer lesions. Tissue sections from intestinal (*a–c*) and diffuse (*d–f*) subtypes of gastric cancer were incubated with pAbs against uPAR together with mAbs against CKs (double staining in *d–f*) or together with mAbs against CKs and mAb against CD68 (triple staining in *a–c*). For double immunofluorescence analysis (*d–f*), the pAbs against uPAR were detected with Cy3-conjugated goat anti-rabbit (red fluorescence) and the mAbs against CKs with FITC-conjugated goat anti-mouse (green fluorescence). For triple immunofluorescence analysis (*a–c*), uPAR pAbs were detected with Cy3-conjugated goat anti rabbit (red fluorescence) together with mAbs against CKs (green fluorescence) and mAb against CD68 (blue fluorescence) using Zenon Antibody Labelling Technology. In gastric cancer tissue, uPAR-positive cancer cells are seen in both intestinal (*a–c*; white arrows) and diffuse (*d–f*; white arrows) subtypes. Several CD68-positive macrophages located in the invasive area in close proximity to cancer cells were strongly uPAR-positive (*a–c*; blue arrows). Nomarsky DIC imaging technique was used in *f*. Scale bar: ~10 μm (a-*f*).

uPAR immunoreactivity in the Norwegian cases was found in all 23 cases, with a pattern of expression indistinguishable to that observed in the cases from Costa Rica. In 13 of the 23 cases from Norway (56%), uPAR-positive cancer cells were observed, with similar frequency of intestinal and diffuse subtype cases showing uPAR-positive cancer cells (p = 0.67; χ^2 = 0.18) and no significant association between uPAR immunoreactivity in cancer cells and tumor stage was demonstrated (p = 0.89; Table 1). Analysis of overall survival of the Norwegian patients suggested that uPAR-positive cancer cells could be a prognostic variable (Cox regression analysis, Hazard ratio = 1.6; 95% CI: 0.5–5) and although not significant, the sample size was very low. We found no statistically significant differences when we compared the frequency of Costa Rican versus Norwegian cases of intestinal subtype gastric cancers showing uPAR-positive cancer cells (p = 0.93; χ^2 = 0.01) or of diffuse subtype gastric cancers with uPAR-positive cancer cells (p = 0.86; χ^2 = 0.03; Table 1). When the category "less than 1% uPAR + cancer cells" is not considered as positive (Table 1), the proportion of intestinal and diffuse subtype cases showing uPAR-positive cancer cells remain similar in both countries (p = 0.89; χ^2 = 0,02 Costa Rica; p = 0.26; χ^2 = 1.25 Norway), and the frequency of Costa Rican versus Norwegian intestinal (p = 0.65; χ^2 = 0.2) and diffuse subtype gastric cancers with uPAR-positive cancer cells (p = 0.48; χ^2 = 0.49). Accordingly, no significant association between uPAR immunoreactivity in cancer cells and tumor stage was demonstrated in any of the countries (p= 0.98 Costa Rica; p = 0.72 Norway).

H. pylori associates with the induction of uPAR in gastric mucosa

Analysis of uPAR expression in non-neoplastic mucosa with some degree of inflammation showed uPAR immunoreactivity in gastric epithelial cells located toward the gastric lumen in the Costa Rican material (Figs. 4*a* and 4*d*). Because a possible relation between *H. pylori* and induction of uPAR expression in gastric cancer cells has been suggested,[30,32] we

Table 1. uPAR immunoreactivity of cancer cells in cases from costa rica and norway according to histological subtypes and tumor stage

	Costa Rica	Norway	Total
Total number of cases	44	23	67
Intestinal subtype	27	15	42
Diffuse subtype	17	8	25
Cases with uPAR + cancer cells	24	13	37
Intestinal subtype cases with uPAR + cancer cells	14	8	22
<1% uPAR + cancer cells	5	4	9
1–5% uPAR + cancer cells	2	3	5
5–10% uPAR + cancer cells	3	0	3
>10% uPAR + cancer cells	4	1	5
Diffuse subtype cases with uPAR + cancer cells	10	5	15
<1% uPAR + cancer cells	4	1	5
1–5% uPAR + cancer cells	0	2	2
5–10% uPAR + cancer cells	3	0	3
>10%uPAR + cancer cells	3	2	5
Cases with uPAR + cells according to tumor stage[1]			
IA	6	0	6
IB	1	1	2
II	3	3	6
IIIA	3	0	3
IIIB	2	0	2
IV	4	9	13
Unknown[2]	5	0	5

[1]The total distribution of cases according to tumor stage is given in the Material and methods section.
[2]Not possible to classify because pN was missing on the records.

explored the possibility that uPAR is upregulated in gastric epithelium as a consequence of *H. pylori* infection. Samples from nonneoplastic mucosa were stained for *H. pylori*. Immunoperoxidase staining showed *H.pylori* in 77% (34 of 44) of the Costa Rican and 30% (7 of 23) of the Norwegian cases ($p = 0.00021$; $\chi^2 = 13.71$). *H. pylori* clusters were observed at the luminal space of the crypts, in direct contact with epithelial cells of the surface and deeper areas of the crypts, and inside some of the mucosal glands (Figs. 4*e* and 4*f*), with the highest density of bacteria in foci of gastritis-affected mucosa (Fig. 4*f*). Immunoperoxidase staining for uPAR in adjacent tissue sections of non-neoplastic mucosa from the 23 Norwegian cases (Table 2), showed expression of uPAR in surface epithelial cells located toward the gastric lumen, similar to our observation in the Costa Rican material (Figs. 4*a*–4*d*). The presence of uPAR on surface epithelial cells was significantly associated with the presence of *H. pylori* ($p = 0.033$; $\chi^2 = 4.54$). Indeed, double immunofluorescence analysis for uPAR and *H. pylori* in tissue sections of non-neoplastic tissue adjacent to the neoplastic growth from 8 of the patients with gastric cancer from Costa Rica (5 *H. pylori*-positive and 3 *H. pylori*-negative), showed intense uPAR immunoreactivity in surface epithelial cells located at the luminal edge of the crypts that were in direct contact with bacteria in the *H. pylori*-positive patients (Figs. 4*h* and 4*i*). In contrast, the *H. pylori*-negative cases showed no or weak uPAR expression in epithelial cells at similar location (not shown).

Discussion

In this study, we show that uPAR is expressed in a part of cancer cells in approximately 50% of all gastric cancers in both high- and low-risk countries. Our findings suggest the further evaluation of uPAR as a potential immunohistochemical parameter, which, based on the prevalence of uPAR-positive cancer cells in the tumor, may provide prognostic information about the patient. Our observations are based on immunohistochemical analyses of 2 uPAR mAbs (R2 and R4) directed against 2 different domains on uPAR, and a preparation of affinity purified polyclonal antibodies against uPAR.[35,36] The 3 different antibody preparations identified uPAR on gastric cancer cells and stromal cells to the same extent, while a mAb directed against the TNP hapten or non-immune rabbit IgG showed no specific staining. We therefore conclude that the uPAR staining observed represent the genuine uPAR protein.

The assumption that uPAR-positive gastric cancer cells are of particular aggressive character is based on analyses of

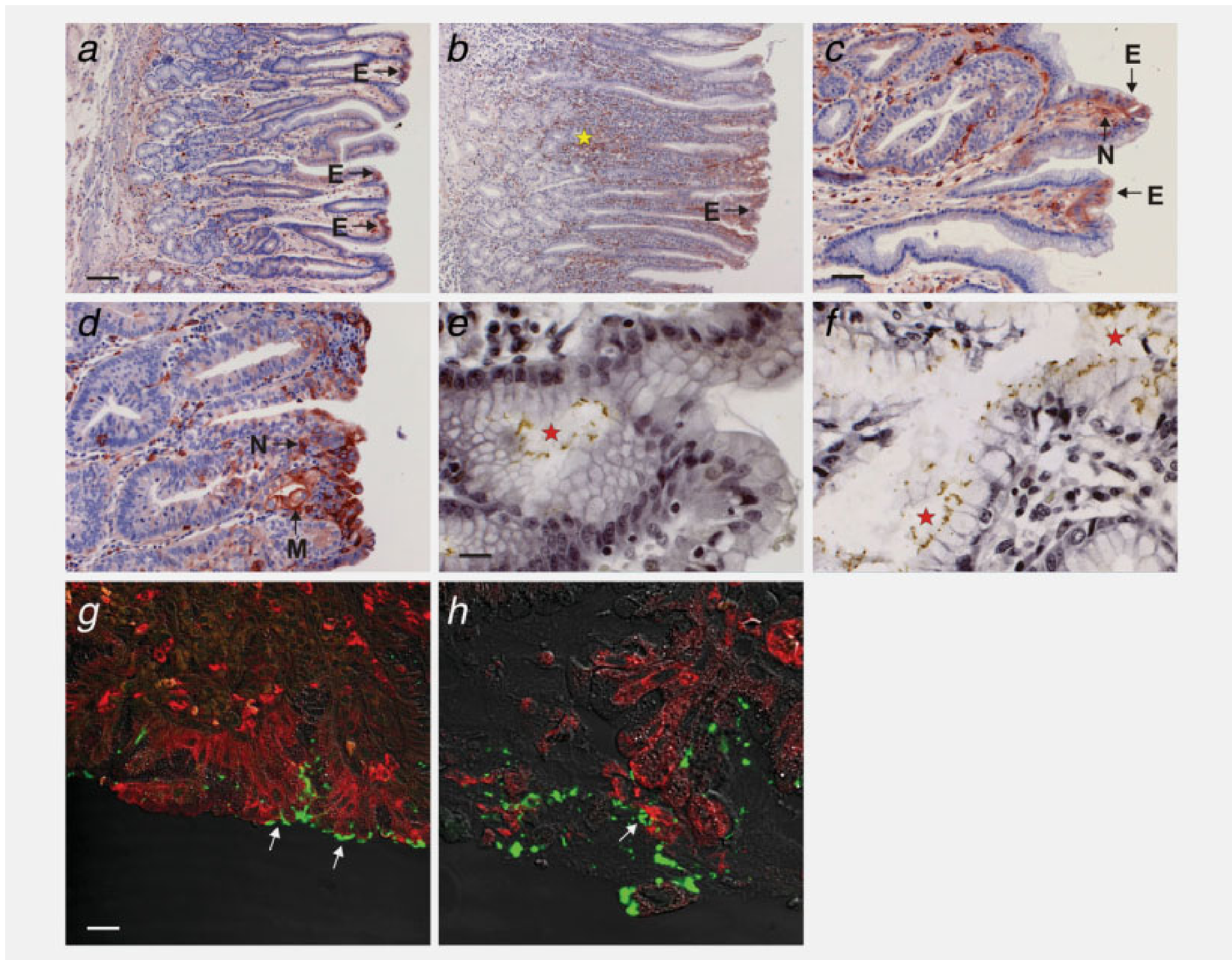

Figure 4. Immunohistochemical analyses of uPAR and *H. pylori* in non-neoplastic gastric mucosa. Sections from samples with gastric mucosa adjacent to the neoplastic lesion were processed for immunoperoxidase staining for uPAR (*a–d*), *H. pylori* (*e–f*) or for immunofluorescence analysis (*g–h*) using pAbs against uPAR, pAbs against *H. pylori* and uPAR mAb R4 together with *H. pylori* pAbs, respectively. In normal-like mucosa (*a*), uPAR is expressed by epithelial cells (E) located at the apical part of gastric mucosa, scattered neutrophils and some inflammatory cells in the *lamina propia*. In gastritis-affected mucosa, uPAR is seen in neutrophils (N) (*b*; star, *d*) and epithelial cells (E) (*b–c*) more evidently than in normal-like foci. Scattered uPAR-positive macrophage-like cells (M) can be observed in gastritis-affected mucosa (*d*). *H. pylori* clusters (red stars) can be observed at the luminal side in some of the crypts along the gastric mucosa (*e*) with the highest density in gastritis-affected mucosa (*f*). In the double immunofluorescence analysis R4 mAb against uPAR together with pAbs against *H. pylori* (double staining in *g–h*) were detected with Cy3-conjugated goat anti-mouse (red fluorescence) and FITC-conjugated goat anti rabbit (green fluorescence), respectively. Intense uPAR immunoreactivity is seen in epithelial cells in direct contact with *H. pylori* bacteria (*g–h*; white arrows). Scale bars: ~100 μm (*a–b*), ~50 μm (*c–d*), ~12 μm (*e–f*), ~20 μm (*g–h*).

Table 2. uPAR immunoreactivity in apical epithelial cells in relation to the *H. pylori* status in non-neoplastic tissue sections from norway

	uPAR scoring				
	0	1+	2+	3+	Total
H. pylori positive	2	1	3	1	7
H. pylori negative	14	1	1	0	16

micro-metastatic cells identified in bone marrow aspirates from patients with gastric cancer,[27] and its significant association with survival.[29] We found uPAR-positive gastric cancer cells with the same frequency in intestinal and diffuse subtypes. In their study, Heiss *et al.*[29] found no significant correlation between the presence of uPAR-positive micro-metastatic cells in bone marrow aspirates and Laurén's classification, meaning that these cells were observed with similar frequency in both intestinal and diffuse subtype. Thus, an accurate determination of the prevalence of uPAR-positive gastric cancer cells by immunohistochemistry could be of fundamental importance owing to its potential prognostic significance and the repercussions that this may have for

the treatment and survival of patients with gastric cancer. We are already working on a larger and independent study to evaluate the usefulness of uPAR immunoreactivity of gastric cancer cells as prognostic variable.

In this study, we found uPAR-positive cancer cells in more than 50 percent of the cases with similar frequency in intestinal and diffuse subtypes of gastric cancer. In contrast, Migita *et al.*[15] observed uPAR-positive cancer cells in only 16 cases of 78 intestinal subtype gastric cancers and in none of diffuse subtype gastric cancers. A methodological difference in the immunohistochemical staining of uPAR between Migita *et al.*[15] and ours is the proteolytic pre-digestion step. Migita *et al.*[15] used trypsin, whereas we used proteinase-K. Epitope demasking is a crucial step for most immunohistochemical analyses. We have observed that the efficiency of the proteolytic predigestion strongly influence the intensity of uPAR immunoreactivity (Alpízar-Alpízar and Nielsen, unpublished observations). It cannot be excluded, however, that geographical differences could explain the differences (see also below). Kawasaki *et al.*[19] used *in situ* hybridization and found uPAR mRNA expression in cancer cells in both intestinal and diffuse subtypes (5 of 33 cases and 14 of 58 cases, respectively).

The strongest contribution to uPAR immunoreactivity in the gastric cancer samples was in general the stromal cell population, particularly the inflammatory cells, macrophages and neutrophils. These cell populations are generally found to be uPAR-positive in a number of tissue types.[14,39] Immunohistochemical analysis of uPAR has therefore appeared to be quite complex; not only in gastric cancer but also in breast, oral and colon cancer, where double immunofluorescence analyses have been crucial for a sufficient discrimination of the different uPAR-positive cell populations.[12,13,16] uPAR-positive cancer cells are found in different types of carcinomas with varying occurrence including colon, breast and oral cancer.[12,13,16] In colon cancer, the uPAR-positive cancer cells are located at the invasive front[13,40] as they also are in the gastric cancers of intestinal subtype ([15] and this article), suggesting similar invasion mechanisms in these 2 gastrointestinal adenocarcinomas. Expression of uPAR in both cancer cells and stromal cells contribute to the total level of uPAR in the cancer tissue. However, it is currently unknown to which extent uPAR derived from the different cell populations contribute to the prognostic significance of uPAR and the uPAR degradation products that can be measured in blood and tissue extracts.[41]

It is believed that uPAR expression on cells facilitate cell migration by interacting with extracellular matrix components like vitronectin,[42,43] or with other cell surface associated proteins, like integrins.[44] Therefore, expression of uPAR on cancer cells is likely to enhance its potential to invade and metastasize. In a metastasis model, transgene overexpression of uPAR in tumor cells confers enhanced proliferative and metastatic potential dependent on the interaction of uPAR with integrins and fibronectin.[45–50] These observations might explain the correlation found between the presence of uPAR-positive micro-metastatic cancer cells in the bone marrow and poor prognosis in patients with gastric cancer.[29,51] Hence, the clinical relevance of uPAR expression in neoplastic cells of gastric cancer may be based on its proinvasive, prometastastatic and proliferative properties.

Gastric cancer incidence presents important variations according to geographical regions.[1] The factors explaining these variations remain unknown so far given the complexity and multifactorial nature of the disease. These different incidence rates are likely to be explained by ethnical, environmental, cultural and socioeconomic aspects. For example, studies assessing the association between genetic polymorphisms of pro and anti-inflammatory cytokines and gastric cancer have shown different results depending on geographical regions.[52–54] Likewise, differences between *H. pylori* strains among geographical regions correlate well with the distribution of the gastric cancer incidence.[33] This suggests that when studying aspects potentially related with gastric cancer development and progression, geographical regions are important to take into account. In this study, we determined the expression of uPAR in cancer cells, in gastric cancer tissue obtained from a country with one of the highest gastric cancer incidence rates worldwide (Costa Rica) and one with low incidence rate (Norway).[7] We did however find similar frequency of uPAR-positive cancer cells in Costa Rican and Norwegian gastric cancer cases, suggesting that the expression of uPAR and its potential role in gastric cancer development and progression is independent of geographical, ethnical or environmental differences. Though, it cannot be excluded that gastric cancer developing in other geographical regions could present different uPAR expression patterns.

Another important finding in this study was the intense uPAR immunoreactivity in epithelial cells located at the luminal edge of the non-neoplastic mucosa. Epithelial cells at this location can, eventually, be in direct contact with *H. pylori* bacteria, and this organism is associated with several responses in the gastric mucosa.[55,56] A semiquantitative analysis of *H. pylori*-positive cases and the presence of uPAR-positive surface epithelium revealed a significant association, and indeed we found *H. pylori* clusters in close proximity to uPAR-positive surface epithelium by double immunofluorescence analysis. *In vitro* studies have shown increased levels of both uPAR mRNA and uPAR antigen when gastric cancer cell lines are challenged with *H. pylori*,[32,57] and when cocultured with *H. pylori* cagA-positive versus negative strains.[31,58] These observations taken together suggest that *H. pylori* may be directly involved in the induction of uPAR in the gastric mucosa. It is tempting to speculate that *H. pylori* can stimulate the expression of uPAR in epithelial cells, which under certain circumstances could potentiate the development of gastric pathological conditions that leads to gastric neoplasia. The findings in a mouse model where simultaneous transgene overexpression of uPAR and uPA in mouse skin confer a pathological epidermal phenotype[59] and the enhanced proliferation potential of uPAR-overexpressing transgenic cell lines,[45–50] support this idea. The final outcome may, however,

result from the interaction of uPAR with other molecules in the mucosal microenvironment including uPA, extracellular matrix proteins and mediators of inflammation released in response to *H. pylori* infection. Hence, the possible connection between *H. pylori* and uPAR and its potential implications in gastric cancer development and progression need to be further explored. These findings raise new and exciting questions regarding the role of *H. pylori* and uPAR in the development of gastric cancer, including elucidation of the role of the various *H. pylori* virulence factors in the induction of uPAR and the potential prognostic value of uPAR in gastric cancer.

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