The immunoexpression of Shh, Smo and Gli2 in *Helicobacter pylori* positive and negative gastric biopsies

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The Hedgehog signaling pathway plays a principal role during embryonic development, tissue regeneration and carcinogenesis in various adult tissues. Although hedgehog signaling is important in gastric carcinogenesis, its role in *Helicobacter pylori*-associated gastritis is unclear. The aim of our study was to examine Sonic Hedgehog (Shh) signaling pathway in response to *H. pylori* infection. Thirty-one formalin-fixed, paraffin-embedded tissue specimens of chronic gastritis were retrieved from archival material. The immunoexpression of Shh, Smoothened (Smo) and Glioblastoma transcription factor 2 (Gli2) were detected using the immunohistochemical method. Sonic Hedgehog protein was expressed in *H. pylori*-positive and *H. pylori*-negative groups of patients. The immunoexpression of Shh, Smo and Gli2 proteins was lower in *H. pylori*-positive group compared to *H. pylori*-negative group, however only the differences in Shh and Smo immunoexpression were statistically significant. The immunoexpression of Shh was significantly correlated with the immunoexpression of Smo in both tested groups ($p < 0.001$, $p < 0.02$, respectively). No statistically significant correlation was found between Shh and Gli2 among *H. pylori*-positive and *H. pylori*-negative groups. The above findings support the hypothesis of the involvement of Shh signaling pathway in *H. pylori*-associated gastritis.

Key words: Shh, Smo, Gli2, *Helicobacter pylori*, gastritis.

Introduction

The Hedgehog (Hh) signaling pathway is one of the key regulators of animal development. The Hh pathway plays a critical role in embryonic development and carcinogenesis in various adult tissues. Recent studies point to the role of Hh signaling in regulating adult stem cells involved in maintenance and regeneration of adult tissues [1-3]. The hedgehog is a secreted protein that is highly conserved from flies to mammals (meaning it was present in the common ancestor of both). There are three members of the mammalian Hh family: Sonic (Shh), Indian (Ihh), and Desert (Dhh) [4]. Sonic hedgehog is the best studied ligand of the vertebrate pathway. The Shh is crucial for the development of various embryonic tissues, including neurons, smooth muscle tissue, bones, epithelial cells, and hematopoietic cells [5-7]. Shh-deficient mice exhibit embryonic lethality with skeletal abnormalities, defects in neuronal development and small body size [8]. The human Shh pathway is engaged in direct tumorigenic actions in various cancers, e.g. basal and squamous cell carcinomas [9, 10], breast [11], prostate [12], lung [13], hepatocellular [14], esophageal [15], gastric [16] and colorectal cancers [17]. Interestingly, all of these cancers arise from the tissues where Shh signaling plays an important developmental role.

The intracellular mechanisms that mediate the action of Shh on target cells have been only partially un-

derstood. According to the recent investigations, Shh is synthesized in epithelial cells. Shh ligands bind to a 12-transmembrane receptor called Patched (Ptch; Ptch1, Ptch2), expressed in adjacent mesenchymal cells. Ptch1 restricts Hh signaling by inhibiting a 7-transmembrane receptor Smoothened (Smo). In the absence of Shh, Ptch1 suppresses the activity of Smo by binding to Smo. Upon Shh stimulation, Shh binds to Ptch1, the inhibition on Smo is removed and processed into forms of Glioblastoma transcription factor protein family (Gli). Gli (Gli1-Gli3) encodes transcription factors and transfers the signals into the nucleus to bind Hh target genes [18-20]. Disruption of *Gli2* or *Gli3* in mice results in severe skeletal and neural defects and embryonic or perinatal lethality [21, 22]. Double mutation of *Gli1* and *Gli2* causes more severe developmental defects than loss of *Gli2* alone [23, 24]. These findings suggest that Shh signaling is critical for normal development and that *Gli1* and *Gli2* have overlapping functions. This hypothesis was confirmed directly by substitution of *Gli1* into the *Gli2* locus [25]. It is well established that Glis are often overexpressed in human cancers. Numerous data suggest that Glis contribute to the progression of a variety of neoplasms via regulation of cell cycle progression and apoptosis [26, 27]. *Gli2* seems to be an important effector of Shh signaling, because disruption of the *Gli2* gene leads to developmental defects involving several Shh target tissues, while *Gli1* null mice are born without detectable abnormalities [26, 28].

Helicobacter pylori (*H. pylori*) infection is globally widespread and a major cause of chronic atrophic gastritis with persistent infection in 50% of the global population [29, 30]. Chronic inflammation caused by persistent *H. pylori* infection is directly linked to the development of precancerous alterations and gastric cancer. Histopathological studies of the gastric mucosa revealed a series of lesions, which apparently represent a continuum of changes from the normal epithelium to carcinoma. This includes, in order of increasing severity – superficial gastritis, chronic gastritis, chronic atrophic gastritis, intestinal metaplasia, and dysplasia [31, 32].

The Hh signaling pathway is essential to gastrointestinal tract development and disruption of the Shh signaling pathway is associated with gastrointestinal tumorigenesis. The evidence suggesting a pivotal role of Shh in the development of stomach comes from Shh-null mice that show an intestinal rather than gastric-type mucosa [33]. Van den Brink *et al.* [34] described that pharmacological inhibition of the Shh signaling pathway in the adult stomach causes gastric atrophy (loss of parietal cells) and subsequent disruption of glandular differentiation. However, the question of the mechanism by which *H. pylori* infection induced Shh cascade of intracellular events, leading to mucosal changes, remains unanswered.

Therefore, the aim of our study was to evaluate an immunoexpression of Shh, Smo, and Gli2 in *H. pylori* positive and *H. pylori* negative groups of chronic gastritis. Another purpose was to found whether immunoexpression of Shh could correlate with immunoexpression of Smo and Gli2 in both tested groups.

Material and methods

Patients

Thirty-one formalin-fixed, paraffin-embedded tissue specimens of chronic gastritis were retrieved from archival material (Chair of Pathomorphology, Medical University of Lodz). The main criteria of tissue selection were histopathological similarities within the group (inflammation type, inflammation intensity and atrophy).

According to the clinical information, biopsies for histopathological assessment were taken from the lesser curvature of the antrum ~1 cm from the pylorus, from the greater curvature of the antrum ~1 cm above pylorus, from a midportion of lesser curvature of the antrum, from the lesser curvature of the antrum immediately below the incisura, and from the middle corpus ~2 cm from lesser curvature. All archival material was routinely fixed in 10% neutral-buffered formalin and embedded in paraffin. Formalin-fixed, paraffin-embedded tissue sections were stained with hematoxylin and eosin (H&E stain) and the histological diagnoses were established according to the current classification. Additionally, all examined cases were stained with Giemsa stain to define *H. pylori* infection. Patients were defined as positive for *H. pylori* if Giemsa test was positive. The selected cases consisted of nineteen *H. pylori*-positive and twelve *H. pylori*-negative cases. The age range for *H. pylori*-positive group was from 42 to 77 years (mean 55.25 years) and for *H. pylori*-negative group – from 27 to 78 years (mean 55.54 years).

Immunohistochemistry

Paraffin-embedded tissue sections were mounted onto SuperFrost slides, deparaffinized, then treated in a microwave oven in a solution of TRS (Target Retrieval Solution, Dako) for 30 minutes (2×6 minutes 360 W, 2×5 minutes 180 W, 2×4 minutes 90 W) and transferred to distilled water. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide in distilled water for 30 minutes, and then sections were rinsed with Tris-buffered saline (TBS, Dako, Denmark) and incubated for 1 hour with primary rabbit polyclonal antibody against: Shh (abcam; ab 73958; dilution 1 : 100), Smo (abcam; ab 72130; concentration 10 μg/ml), and Gli2 (abcam; ab 26056; concentration 6 μg/ml). Immunoreactive proteins were visualized using EnVision-

horseradish peroxidase kit (Dako, Carpinteria, CA, USA) according to the instructions of the manufacturer. Visualisation was performed by incubation of the sections in a solution of 3,3'-diaminobenzidine (DakoCytomation, Denmark). After washing, the sections were counter-stained with hematoxylin and cover-slipped. For each antibody and for each sample, a negative control was processed. Negative controls were carried out by incubation in the absence of the primary antibody and always yielded negative results.

In each specimen, staining intensity of Shh and Smo was recorded semiquantitatively by two independent observers in 7-10 adjacent high power fields and graded 0 (staining not detectable), 1 (weak immunostaining), 2 (moderate immunostaining intensity) and 3 (strong staining). The mean grade was calculated by averaging grades assigned by two authors and approximating the arithmetical mean to the nearest unity.

Morphometry

Histological morphometry was performed by means of an image analysis system consisting of a PC computer equipped with a Pentagram graphical tablet, Indeo Fast card (frame grabber, true-color, real-time), produced by Indeo (Taiwan), and color TV camera Panasonic (Japan) coupled to a Carl Zeiss microscope (Germany). This system was programmed (MultiScan 8.08 software, produced by Computer Scanning Systems, Poland) to calculate the number of objects (semiautomatic function).

The colored microscopic images were saved serially in the memory of a computer, and then quantitative examinations were carried out. The percentage of nuclei expressing Gli2 antigen was estimated by counting 100 cells in ten monitor fields (0.0205 mm^2 each), marking immunopositive cells, so that in each case 1000 cells were analyzed.
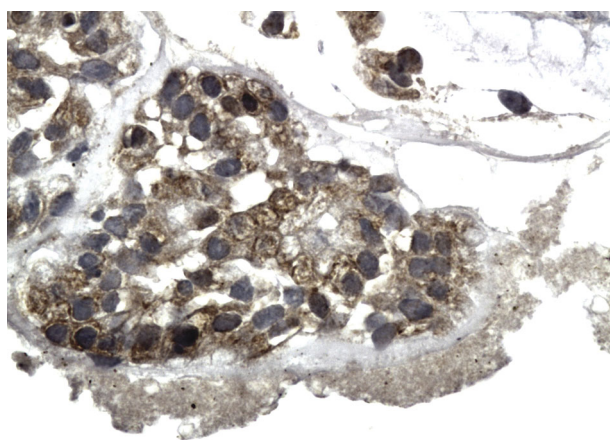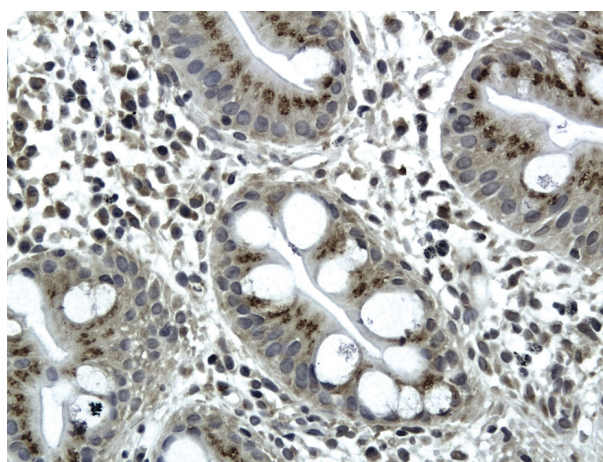
Statistical analysis

All values were expressed as the mean $\pm$ SD (standard deviation). The differences between groups were tested using Student t-test for independent samples preceded by evaluation of normality and homogeneity of variances with Levene's test. Additionally, the Mann-Whitney U test was used where appropriate. Correlation coefficients were calculated using Spearman's method. Results were considered statistically significant if $p < 0.05$.

Results

The results of immunohistochemistry showed that Shh, Smo and Gli2 staining was detected in the glandular epithelial cells and in the stroma of the stomach. Shh immunoexpression was strongly detected in the cytoplasm (Fig. 1 and Fig. 2), Smo was expressed in both the cytoplasm and membrane (Fig. 3 and Fig. 4) and Gli2 protein was expressed in nuclei of epithelial cells (Fig. 5 and Fig. 6). All examined proteins were expressed in *H. pylori*-positive and *H. pylori*-negative groups of patients. The semiquantitative data of immunoexpression of Shh and Smo as well as morphometric data of Gli2 immunoexpression in *H. pylori*-positive and *H. pylori*-negative groups are given in Table I. The immunoexpressions of Shh, Smo and Gli2 proteins were lower in *H. pylori*-positive group as compared to *H. pylori*-negative group, however only the differences in Shh and Smo immunoexpression were statistically significant ($p < 0.03$ and $p < 0.001$, respectively).

The immunoexpression of Shh was significantly correlated with the immunoexpression of Smo in both tested groups ($p < 0.001$, $p < 0.02$, respectively). No statistically significant correlation was found between Shh and Gli2 among *H. pylori*-positive and *H. pylori*-negative group ($p = 0.26$, $p = 0.55$, respectively) (Table II).

Fig. 1. Intense immunoexpression of Shh in glandular epithelial cells of a *H. pylori*-negative patient. Immunohistochemistry. Magnification 400×
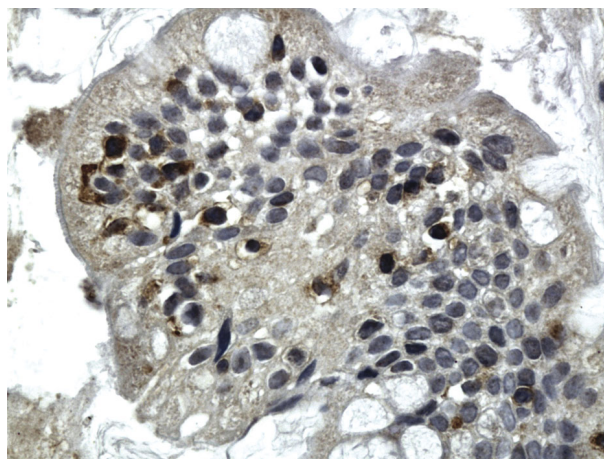

Fig. 2. Delicate immunoexpression of Shh in glandular epithelial cells and in the stroma in a *H. pylori*-positive case. Immunohistochemistry. Magnification 200×
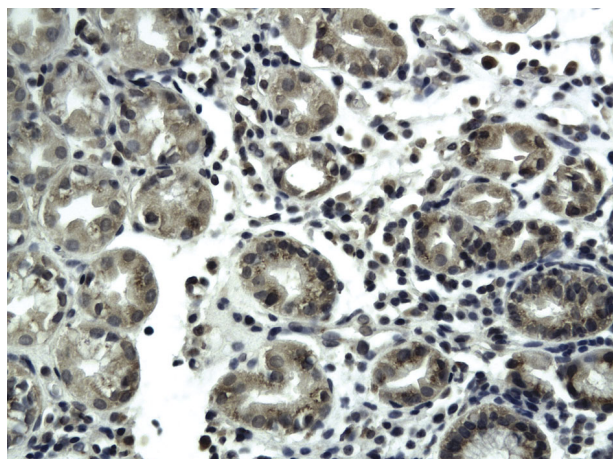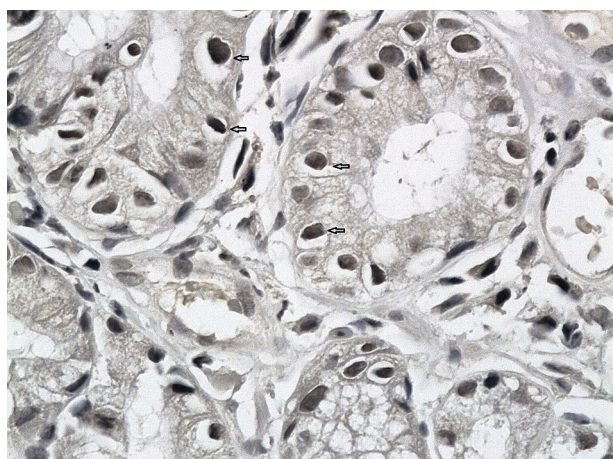
Fig. 3. Strong cytoplasmic and weak membrane immunoexpression of Smo in glandular epithelial cells and in the stroma of a patient from *H. pylori*-negative group. Immunohistochemistry. Magnification 400×

Fig. 4. Weak immunoexpression of Smo in the epithelium of a *H. pylori*-positive case. Immunohistochemistry. Magnification 400×

Fig. 5. Nuclear immunoexpression of Gli2 in glandular epithelial cells of a *H. pylori*-negative patient. Immunohistochemistry. Magnification 400×
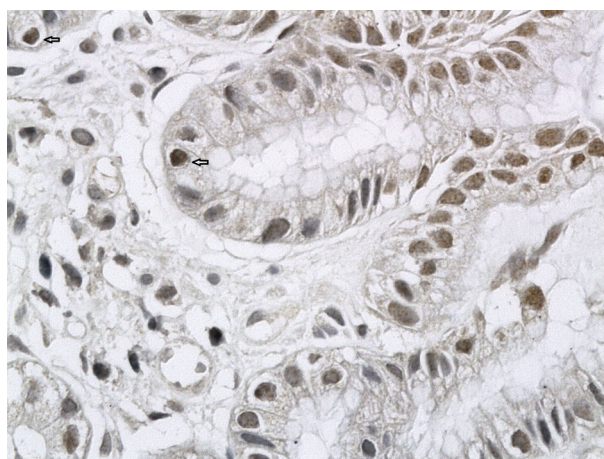
Fig. 6. The immunoexpression of Gli2 in glandular epithelial cells of a *H. pylori*-positive case. Immunohistochemistry. Magnification 400×

Table I. The semiquantitative data of the immunoexpression of Shh and Smo and morphometric data of the Gli2 immunoexpression in *H. pylori*-positive and *H. pylori*-negative groups

	Shh	Smo	Gli2
H. pylori-positive group (n = 19)	1.22 ±0.66	0.98 ±0.41	1.33 ±0.5
H. pylori-negative group (n = 12)	1.75 ±0.49	1.69 ±0.37	1.7 ±0.52
Value of p	< 0.03	< 0.001	0.06 (NS)

Data are expressed as mean ± standard deviation

Table II. Correlations between the immunoexpression of Shh, Smo and Gli2 in *H. pylori*-positive and *H. pylori*-negative groups

Pair of variables	*H. pylori*-positive group (n = 19)	*H. pylori*-negative group (n = 12)
Shh vs. SMO	r = 0.72, p < 0.001	r = 0.68, p < 0.02
Shh vs. Gli2	r = 0.27, p = 0.26 (NS)	r = 0.55, p = 0.06 (NS)

Discussion

It is widely accepted that inflammation that is caused by *H. pylori* infection is a trigger for the development of gastric cancer. During chronic infection with *H. pylori*, Shh is probably implicated in stem/progenitor cell restitution of damaged gastric mucosa [3, 31, 35-37]. Nevertheless, the question of the mechanism by

which *H. pylori* infection induces mucosal lesions remains unanswered.

We examined Shh signaling pathway changes in chronic gastritis in response to *H. pylori* infection. In our study, the immunoexpression of Shh protein was statistically significantly lower in *H. pylori*-positive as compared to *H. pylori*-negative group of chronic gastritis. Shiotani *et al.* [38] also showed that the Shh immunoexpression was significantly lower in *H. pylori*-positive as compared to *H. pylori*-negative cases of atrophic gastritis. Previous studies indicated that loss of Shh immunoexpression correlated with the degree of gastric atrophy and intestinal metaplasia in *H. pylori*-infected patients [38]. A possible explanation for the reduction of Shh immunoexpression in *H. pylori*-associated gastritis is a correlation between loss of Shh immunoexpression and a lower number of parietal cells on morphogenic bases [31, 35-38]. It is well established that the loss of mature parietal cells from the gastric glands of the stomach by either genetic or pharmacological methods results in severe abnormalities in the differentiation and development of gastric cells. These observations underscore the importance of the parietal cells in the regulation of cellular growth and differentiation in the gastric mucosa. Moreover, Shh is almost exclusively expressed in parietal cells of the adult stomach, influencing fundic gland differentiation [35, 39]. Loss of the acid-secreting parietal cells leads to alterations in the cell lineages with expansion of metaplastic mucous cells. In case of gastric atrophy and intestinal metaplasia, where gastric morphogenesis is reduced or absent, the immunoexpression of Shh is reduced or lost.

Correa *et al.* [40] showed that the presence of gastric atrophy is a reliable indicator of preneoplastic changes in the stomach. Shiotani *et al.* [38] suggest that loss of Shh is an early change that occurs in the mucosa prior to neoplastic transformation and loss of Shh may be predicted to be an initial marker for the development of gastric cancer. Recent data suggest that the expression of Shh within the gastric cells is dependent on acid secretion and inflammatory cytokines [35, 38]. Waghray *et al.* [41] using Shh-LacZ mice, demonstrated that proinflammatory cytokine IL-1β reduces the Shh expression and function in the gastric mucosa by reducing acid secretion from parietal cells. These results strongly support the hypothesis that chronically suppressed levels of Shh may eventually reduce the enzyme expression that is sufficient to induce gastric atrophy.

To our knowledge, data concerning Shh signaling pathway related molecule expression in *H. pylori*-associated gastritis are rather scanty. Despite Shh, we examined the immunoexpression of Smo and Gli2 proteins. The immunoexpression of Smo protein was significantly lower in *H. pylori*-positive group as compared to *H. pylori*-negative group. In both tested groups,

the immunoexpression of Shh was significantly correlated with the immunoexpression of Smo indicating that Smo is involved in the Shh signaling pathway.

We also found that the immunoexpression of Gli2 protein was lower in *H. pylori*-positive group as compared to *H. pylori*-negative group of chronic gastritis. In all *H. pylori*-positive and *H. pylori*-negative cases, an elevated immunoexpression of Gli2 was accompanied with an increased Shh immunoexpression, but this correlation in both tested groups did not reach statistical significance. A possible explanation for our results can be a small number of cases and methodological issues, but it is also possible that proteins other than Gli2 may mediate the effect of Shh pathway activation. It is well known that the Gli family plays an important role in the Shh signaling pathway, but the precise role of individual Gli molecules taking part in the signal transduction from Shh protein to Gli in various lesions is still unknown.

In conclusion, although our findings support the hypothesis of involvement of Shh signaling pathway in the pathomechanism of chronic *H. pylori*-associated gastritis, further studies involving a much larger number of cases are needed to better understand the relationship between *H. pylori* infection and Shh signaling pathways.

Acknowledgments

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