Pathol. Oncol. Res. (2011) 17:677–683
DOI 10.1007/s12253-011-9368-9

RESEARCH

Increased Expression of Toll-like Receptors (TLR) 2, 4 and 5 in Gastric Dysplasia

Pedro Pimentel-Nunes · Luís Afonso · Paula Lopes · Roberto Roncon-Albuquerque Jr · Nádia Gonçalves · Rui Henrique · Luís Moreira-Dias · Adelino F. Leite-Moreira · Mário Dinis-Ribeiro

Received: 5 December 2010 / Accepted: 10 January 2011 / Published online: 1 April 2011

Abstract TLRs are important innate immunity receptors. Even though TLR2, 4 and 5 appear to be important for *Helicobacter pylori* (HP) recognition, their role in the evolution of gastritis to more advanced lesions is still unknown. To compare the expression of TLR2, 4 and 5 in normal gastric mucosa, HP+ gastritis, intestinal metaplasia, dysplasia and adenocarcinoma. Immunohistochemistry for TLR2, 4 and 5 was performed with anti-TLR2-TLR4-TLR5 antibodies in 117 histological samples of normal gastric mucosa (n=22), HP+ gastritis (n=20), intestinal metaplasia (n=33), dysplasia (mucosectomy specimens, n=20) and intestinal type adenocarcinoma (surgery specimens,n=22); quantification of expression was performed independently by two pathologists taking into account the percentage of positive epithelial cells and the degree of expression (zero to three score). A statistically significant trend for progressive increase of TLRs expression from normal mucosa to gastric dysplasia was found (mean expression: normal mucosa 0.1; gastritis 1.0; metaplasia 2.2; dysplasia 2.8, p<0.01). All dysplasia samples presented more than 90% positive epithelial cells with strong expression (2.8;95%CI2.7–3). There was less TLRs expression in carcinomas (TLR2:1.0; TLR4:2.0 and TLR5:1.2, p<0.05) when compared with dysplasia, with TLR4 being more expressed than TLR2 and 5 in these lesions (p=0.03). A score of all markers' expression of eight leads to a low (4%) false positive rate in patients with precancerous conditions. Progression of gastric lesions associated with gastric carcinogenesis is associated with increased TLRs expression. Gastric dysplasia presents a high level of TLRs expression, suggesting that these receptors may play a role in adenocarcinoma development.

Keywords Gastric pathology · Dysplasia · Innate immunity receptors · TLRs

P. Pimentel-Nunes (✉) · R. Roncon-Albuquerque Jr · N. Gonçalves · A. F. Leite-Moreira
Department of Physiology, Cardiovascular Research & Development Unit, Faculty of Medicine, University of Porto, Al. Prof. Hernâni Monteiro, 4200-319, Porto, Portugal
e-mail: pedronunesml@gmail.com

P. Pimentel-Nunes · L. Moreira-Dias · M. Dinis-Ribeiro
Gastroenterology Department, Portuguese Oncology Institute, Porto, Portugal

L. Afonso · P. Lopes · R. Henrique
Department of Pathology, Portuguese Oncology Institute, Porto, Portugal

R. Henrique
Department of Pathology and Molecular Immunology, Institute of Biomedical Sciences Abel Salazar, University of Porto, Porto, Portugal

M. Dinis-Ribeiro
CINTESIS/Department of Biostatistics and Medical Informatics, Faculty of Medicine, University of Porto, Porto, Portugal

Introduction

The innate immune system by recognizing several conserved microbial antigens is the first line of defense against infection, initiating in this way protective immunological responses [1, 2]. The toll-like receptors (TLRs) are the most important class of pathogen-associated molecular patterns (PAMPs) receptors, with ten different TLRs being ubiquitously expressed in humans [2–5]. TLRs are membrane-surface receptors consisting of a distinct leucine-rich repeat (LRR) extracellular domain that confers specificity to the receptor, and a conserved toll/interleukin 1 (IL1) receptor

(TIR) intracellular domain [3, 4]. Of the several identified TLRs described, the TLR2, TLR4 and TLR5 subtypes are critically involved in immune responses to bacterial infections, being abundantly expressed in immune cells [6, 7]. In general, TLR2 recognizes PAMPs mainly from Gram positive bacteria, TLR4 is the receptor for Gram negative bacteria lipopolysacharide (LPS) and TLR5 recognizes bacterial flagellin [2, 8].

Helicobacter pylori (HP) is a Gram-negative bacterium that adheres to the surface of gastric mucosa, causing marked inflammation without invasion of gastric epithelial cells [9]. It is believed that HP is a major risk factor for intestinal-type gastric cancer. By promoting a chronic gastric inflammatory state, HP appears to initiate a carcinogenesis sequence that involves chronic gastritis, intestinal metaplasia (IM), gastric dysplasia and, finally, intestinal-type gastric adenocarcinoma [10–15]. However, it appears that once this process begins it could be independent of HP status since premalignant lesions such as IM present irreversible genetic alterations that can promote progression to cancer without HP presence [16–18].

It is clear that TLRs are essential for HP recognition and subsequent innate and adaptive immunity against this bacterium [19]. Several TLRs may play a role in gastric immunologic response to HP [19]. TLR2 appears to be the receptor responsible for most of the inflammatory process that occurs as the result of HP infection [19–21]. However, other studies suggest that TLR4 also play an important part in HP infection by recognizing several HP antigens [22–24]. Concerning TLR5, the data are contradictory with some studies suggesting interaction between HP flagellin and this receptor[25, 26], and others demonstrating that TLR5 is unresponsive to HP flagellin [27–29]. Nevertheless, the role of these receptors in gastric carcinogenesis may go beyond HP infection, since they have been associated to different cancers [19].

Despite the importance of TLR in the inflammatory activation to HP infection and in several oncogenic lines, its role in the progression of the lesions associated with gastric carcinogenesis remains largely unknown [19]. In the present study, TLR2, TLR4 and TLR5 expression was evaluated by immunohistochemistry in normal gastric mucosa, chronic gastritis, intestinal metaplasia, gastric dysplasia and in intestinal-type gastric adenocarcinoma in an attempt to better understand the potential role of those receptors in gastric carcinogenesis.

Material and Methods

Participants and Histological Samples

Data base of the institution, year 2004, was searched for all the gastric lesions to be studied. A total of 20 samples per lesion were estimated to be necessary. Samples of normal gastric mucosa (n=22), chronic active HP gastritis (n=20), complete (n=16) and incomplete IM (n=17) were obtained by endoscopy biopsy. Endoscopic mucosectomy tissue specimens (n=20) were considered for investigation of gastric dysplasia and surgical tissue specimens (n=22) were considered for intestinal-type gastric adenocarcinoma. HP was present in 13 IM (39%) samples and in 5 (20%) mucosectomy samples. After selection, all the samples (n=117) were reevaluated and, whenever necessary, reclassified by an independent pathologist.

The study protocol was approved by the Ethics Committee of Portuguese Oncology Institute, Porto.

Immunohistochemistry

Tissue specimens were fixed in 10% neutral buffered formalin for 24 h and paraffin embedded. Deparaffinized tissue slides were submitted to antigen retrieval using a high temperature antigen unmasking technique in a water bath, 95° in citrate buffer pH6.0, for 20 min. Endogenous peroxidase activity was blocked by incubating the slides with freshly prepared 0.5% hydrogen peroxide in distilled water for 20 min. After washing the slides in distilled water and PBS/0.05% Tween 20 solution, immunostaining was performed using an immunoperoxidase method according to de manufacturer's instructions. The slides were incubated with normal horse serum (Vector Laboratories, Burlingame, CA,USA) 1/50 in PBS-bovine serum albumin (BSA) 1% at room temperature for 20 min in humid chamber. Sections were then incubated with primary antibody at 4°C overnight. The following primary antibodies were used: rabbit polyclonal antibody anti-TLR2 (H-175,1:50 dilution, Santa Cruz Biotechnology, California, USA), rabbit polyclonal anti-TLR4 (H-80, 1:100 dilution, Santa Cruz Biotechnology, California, USA) and rabbit polyclonal anti-TLR5 (H-127, 1:100 dilution, Santa Cruz Biotechnology, California, USA). The slides were then rinsed in PBS/0.05% Tween 20 solution, and bound antibody was detected by applying biotinylade secondary antibody (Vectastain Universal Elite ABC Kit) for 30 min. After wash the slides with PBS/0.05% Tween 20 solution the slides were incubated with ABC reagent (Vectastain Universal Elite ABC Kit) for 30 min. The slides were washed in PBS and incubated for 7 min in 3,3-diaminobenzidine (DAB; Sigma-Aldrich, USA) 0.05 g/PBS, 0.03%H_2O_2. Following counterstaining with hematoxylin for 20 s, the slides were washed for 4 min in water, dehydrated and mounted with Entellan (Merck KGaA, Darmstadt, Germany). Normal gastric mucosa and lymph node tissue were used as negative and positive controls, respectively.

Immunohistochemical Evaluation and TLRs Expression Quantification

In order to quantify TLRs expression in tissue samples three parameters were considered: 1. *Sample positivity*: A sample was considered positive if gastric epithelial cells were clearly stained by the antibody. The results were presented as a proportion (positive samples/total samples of a specific lesion); 2. *Grade of expression*: A score of 0 to 3 was considered according to the number of epithelial cells stained (0—no cells; 1—less than 10% of epithelial cells; 2—10–75% cells; 3—more than 75% cells); 3. *Intensity of expression*: A score of 0 to 3 was considered according to a subjective evaluation of the intensity of stained cells (0—no staining; 1—weak positive staining; 2—moderate positive staining; 3—intense positive staining). The mean of the grade with the intensity of expression was considered as the *final expression score*.

All the samples were evaluated and quantified by two independents pathologists.

Statistical Analysis

Data analysis was performed using the computer software Statistical Package for Social Sciences-SPSS for Windows (version 17.0). Data was presented as mean ± 95% confidence interval (95%CI) or as a proportion of positive samples. One way ANOVA and Student's t test for paired and unpaired data (or correspondent non-parametric test) were used, when appropriate, for comparison between groups. To test the difference of positivity among groups a linear-by-linear association for the binary values was used. In order to evaluate the tendency for increase or decrease expression, t test for trend was used. Statistical significance was set at $p<0.05$. Hypothesizing the use of relative expression of TLR2, 4 and 5 to help in the diagnosis of dysplasia or invasiveness, a score was then calculated by the sum of the mean score for each marker, varying between 0 and 9. The best cutoff for the diagnosis of lesions as severe as dysplasia, for the diagnosis of dysplasia and for the diagnosis of invasive cancer were described and estimates of sensitivity and specificity for each outcome calculated.

Results

Positivity of the Samples for TLRs Expression

Figure 1 depict the results of TLR2, 4 and 5 immunoexpression for the different tissue samples. The proportion of positive samples in normal gastric mucosa was very low for all TLRs (5–14%). When HP was present these values were significantly higher (50–75%, $p<0.01$ Vs normal mucosa). Almost all the metaplasia and all dysplasia samples were TLRs positive ($p<0.01$ Vs normal or HP gastritis). When carcinoma was considered, we found a significant decrease of positive samples for TLR2 and TLR5 (55% and 64%, respectively, $p<0.05$ Vs metaplasia or dysplasia) but not for TLR4 (86%, $p=0.08$). This occurred because carcinoma were more frequently positive for TLR4 than for TLR2 or TLR5 ($p<0.05$).

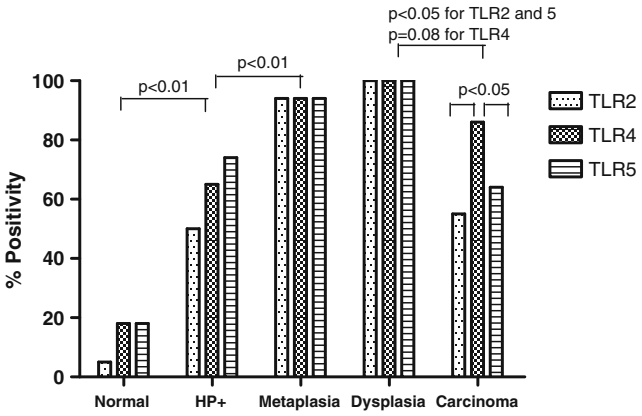

Fig. 1 Proportion of TLRs positive samples (gastric epithelium) in the different gastric lesions

TLRs Expression in the Different Gastric Lesions

In Fig. 2, TLRs expression in the different gastric lesions is shown. Normal gastric mucosa weakly expressed all TLRs (0.15; 95%CI 0.0–0.3). HP gastritis had increased TLRs expression (five to ten fold higher expression for all TLRs, $p<0.001$), still, with a weak expression (mean expression of 10% gastric epithelial cells and weak intensity of expression in the

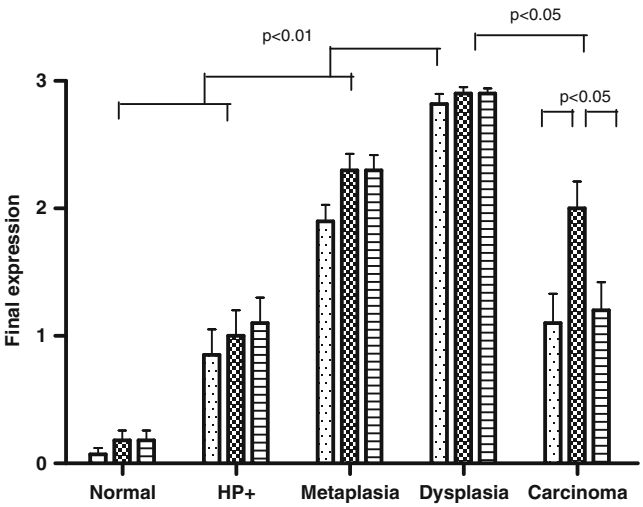

Fig. 2 TLRs final expression (mean and 95%CI) in the different gastric lesions

majority of the samples (1.0; 95%CI 0.6–1.4)). TLRs were strongly expressed in almost all areas of intestinal metaplasia (>75% gastric epithelial cells and moderate intensity of expression (2.2; 95%CI 1.8–2.6)), with no differences between complete or incomplete metaplasia (2.1 vs 2.2, p= 0.8). There were also no differences between IM with or without HP (2.2 vs 2.0, p=0.5). More important, in dysplasia, TLRs expression was maximum in all areas (>90% gastric epithelial cells, strong intensity of expression (2.8; 95%CI 2.7–3)) and clearly superior to all the other gastric lesions (p< 0.01). These results in dysplasia lesions were completely independent of HP status (p=0.9). In intestinal-type adenocarcinoma, some tumors had a high level of TLRs expression in almost all the cells with a strong intensity. Others, however, showed a very weak expression for one or all TLRs. Nevertheless, TLR4 expression in tumors was higher than TLR2 or TLR5 (2.0 Vs 1.0 or 1.2, respectively, p<0.05). There was a statistical significant trend for increase of TLRs expression from normal mucosa to gastric dysplasia (p<0.01).

Cellular Distribution of TLRs

Gastric epithelium of normal mucosa, with or without HP, expressed all TLRs in a polarized manner, particularly at the basolateral membrane but also at the apical membrane (Fig. 3a). On the other hand, metaplasia, dysplastic and neoplastic epithelial cells expressed all TLRs diffusely and homogeneously throughout the cytoplasm with no apparent polarization (Fig. 3b–f).

Score for the Diagnosis of Dysplasia/Cancer Using TLRs Relative Expression

When adding the relative expression of TLR 2,4 and 5, the presence of a score of 1 seems to leads to a very low rate of

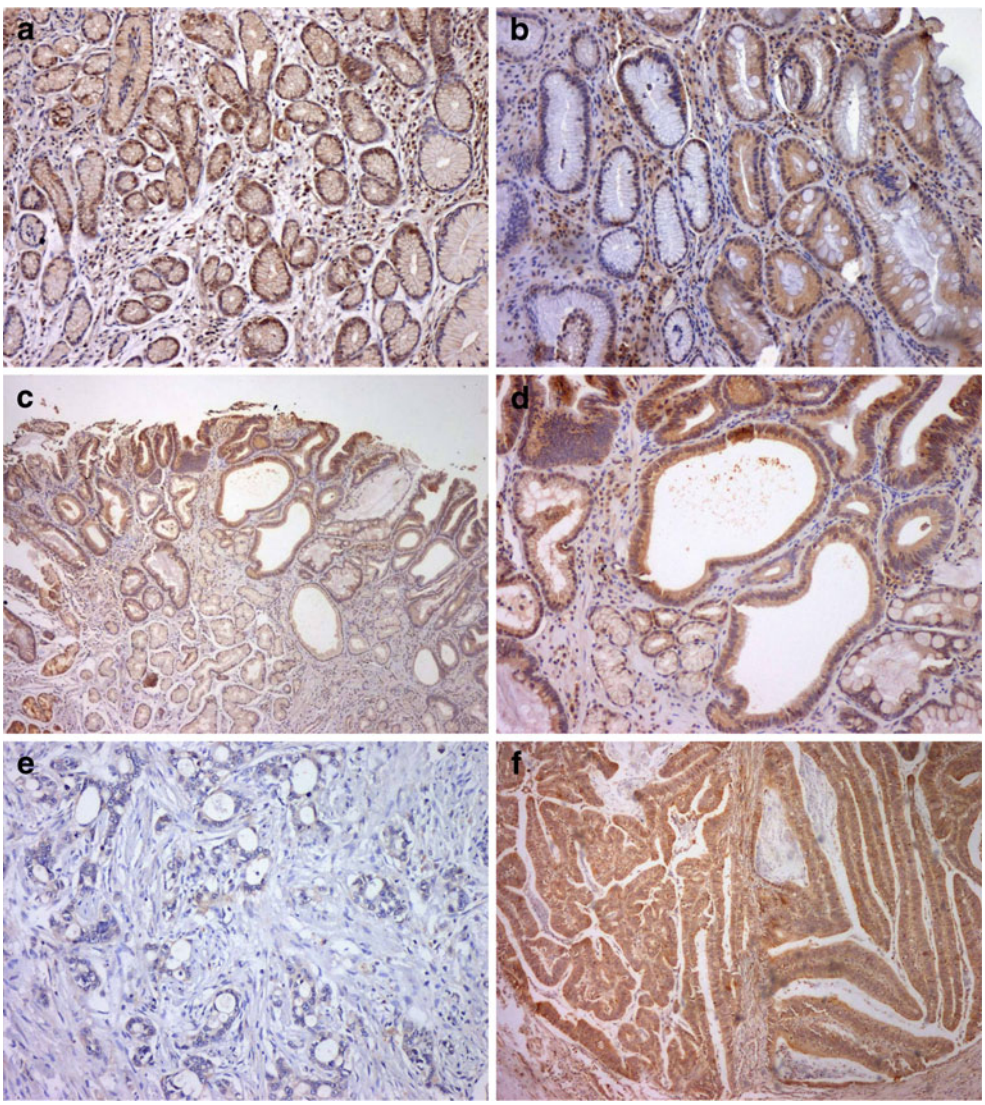

Fig. 3 Immunohistochemistry images of the different lesions. **a** HP+ gastritis—Weak to moderate and polarized expression in this case for TLR2 (similar to the others TLRs); **b** Normal and metaplasia—In the left, normal mucosa with polarized and very weak TLR5 expression, with the transition in the right for intestinal metaplasia with diffusely and moderate to strong TLRs expression; **c** (low power field) and **d** (high power field) Gastric dysplasia—In this mucosectomy specimen we can see the rising levels of TLR4 expression from normal mucosa (*down*), metaplasia (*right*), to dysplasia (*up*) that presents a very strong, diffuse, expression in almost all epithelial cells; **e** and **f** adenocarcinoma—Some tumors presented very weak expression (**e**) and others presented a very strong, diffuse expression (**f**), in this case for TLR2

Table 1 Score for the diagnosis of dysplasia and cancer obtained by adding the relative expression of TLR 2,4 and 5

Diagnosis	N	AUC (95%CI)	Cutoffs	Sensibility	Specificity
At least dysplasia	117	0,75 (0,65-0,85)	1[c]	100	30
			8[c]	40	96
Dysplasia vs other lesions[a]	95	0,95 (0,91-0,99)	1[c]	100	30
			8[c]	75	96
Invasive cancer[b]	42	0,94 (0,00-1,00)	1[d]	100	9
			8[d]	91	75

[a] Patients with invasive cancer were excluded

[b] Among patients with neoplasia

[c] The cutoff presented means that individuals with less than that value in the Cumulative score would be considered as having no outcome (dysplasia), whereas those with the cutoff value or more, would be considered with lesions as severe as dysplasia

[d] The cutoff value means that individuals with less than that value would have invasive cancer

false negative for lesions as severe as dysplasia. To adequately identify dysplasia a score of 8 seems to be very useful as it leads to a very low false positive rate (4%) in patients with precancerous conditions and also to a low false positive rate (missing invasiveness) when distinguishing dysplasia from invasive cancer (Table 1). Figure 4 shows the respective Receiver Operating Curves.

Discussion

In the present study, TLR2, TLR4 and TLR5 immunoexpression was evaluated in gastric lesions associated with gastric carcinogenesis. A significant increase of TLRs expression from normal mucosa to gastric dysplasia was found. Intestinal-type adenocarcinoma also presented significant expression of these receptors, particularly for TLR4.

Previous reports described TLRs expression in gastric lesions [23, 24, 30, 31]. Similar to our study, Schmausser et al. [31] suggested that HP augments TLRs expression in gastric mucosa and that metaplasia and carcinoma had more TLRs expression than normal mucosa. However, owing to a low number of histologic samples, they were unable to quantitatively compare TLRs expression between the different gastric lesions. Our study clearly showed that TLRs present a gradual increase of expression from normal mucosa to gastric dysplasia, with these lesions presenting more than 90% of epithelial cells with strong positivity for these receptors. Moreover, contrarily to normal mucosa, IM and dysplasia lesions presented diffuse positivity of these receptors, which may suggest an easier activation of these receptors.

Some limitations can be pointed to our study. First, quantification of expression was done only by immunohistochemistry. Second, the scores for TLRs expression were

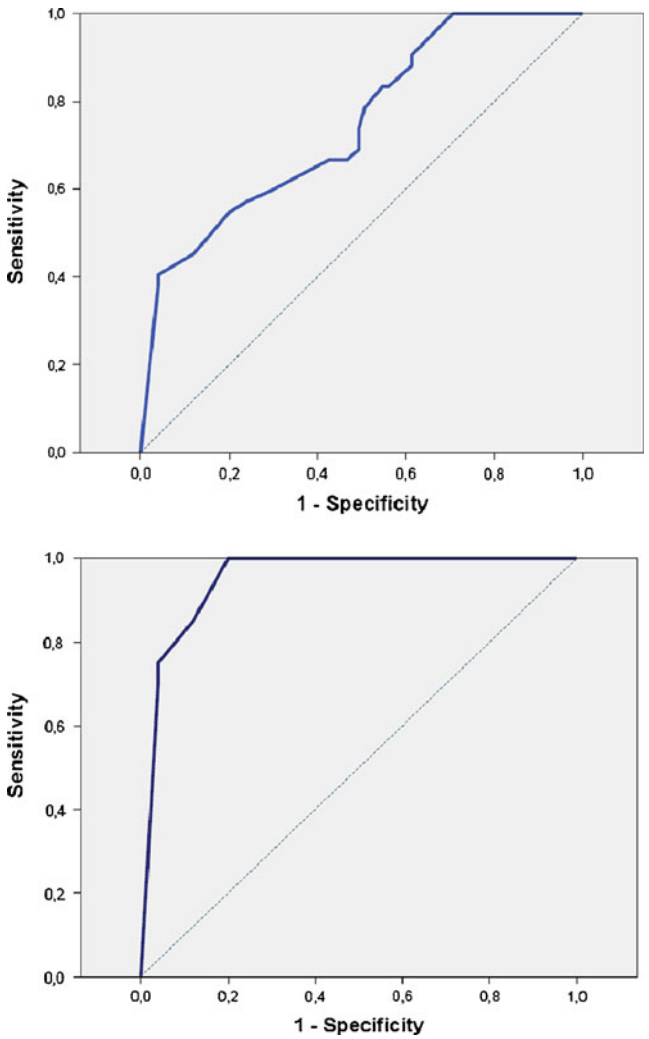

Fig. 4 Receiver operating curves for the diagnosis of neoplastic lesions (*up*) or Dysplasia versus other lesions excluding invasive adenocarcinoma (*down*) using the cumulative score of TLRs expression

subjective. However, samples were evaluated by two independent and expert gastrointestinal pathologists, that come to similar results. On the other way, a strong aspect of our study was the inclusion of mucosectomy samples. In fact, in these samples we clearly observed all the spectrum of gastric lesions, and consistently distinguished the different levels of expression in these lesions, supporting our results. Moreover, we have shown that a score obtained by adding the relative expression of TLR 2,4 and 5 may have diagnostic value since to adequately identify dysplasia a score of 8 was very useful leading to a very low false positive rate (4%) in patients with precancerous conditions and also to a low false positive rate (missing invasiveness) when distinguishing dysplasia from invasive cancer.

What can we learn from these results? In order to maintain gastrointestinal homeostasis it appears that gastric epithelium, similar to colonic epithelium, has a very low expression of TLRs, fundamentally confined to the basolateral membrane [32–34]. HP appears to initiate a cascade that leads to chronic infection and increase TLRs expression in gastric epithelial cells. Chronic infection promotes phenotypic change to gastric IM, which, as we have seen, has a high and diffuse TLRs expression. We can speculate that at this phase the presence of HP is not absolutely necessary for epithelial stimulation. Actually, gastric epithelium is exposed daily to innumerous bacteria that, despite not being able to initiate a gastric infection like HP, have the potential to stimulate these diffusely overexpressed receptors. In fact, some studies show that, when stimulated, these receptors lead to the production of several cytokines and growth factors as well as to an increase in COX-2 expression, conferring an important oncogenic potential to these receptors [35, 36]. Dysplasia, by presenting even more TLRs expression, can accelerate these processes, leading to the development of gastric adenocarcinoma. Confirming the potential role of these receptors in the progression of gastric lesions, some studies associated TLR4 and TLR2 polymorphisms with the severity of gastric lesions [37–41]. Future studies should evaluate if blockage of TLRs can delay progression of lesions and carcinoma development.

Concerning adenocarcinoma, we found that a large number of tumors significantly express these receptors, particularly TLR4. Others showed that TLRs stimulation in gastric tumor cells can induce several gastric carcinoma promoting factors leading to proliferation and progression of gastric cancers [42–44]. Taking together these observations, it is possible that TLRs expression in gastric tumors can influence prognosis and that antagonists of these receptors can have therapeutic value. Future studies should have these aspects in consideration.

In conclusion, progression of gastric lesions associated to gastric carcinogenesis is accompanied by a progressive increase of TLRs expression in gastric epithelial cells. Gastric dysplasia presents a very high level of TLRs expression, suggesting that these receptors may have a role in carcinoma development. Adenocarcinomas also present a significant expression of these receptors, which may influence tumoral progression. Molecular and functional studies are necessary to clarify the role of these receptors in gastric carcinogenesis.

Acknowledgments This study was supported by grants for medical investigation from Portuguese Oncology Institute of Porto.

None of the authors have any disclosure.

Competing interest None to declare.

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