

Immunohistochemical expression of Ki-67 and p53 along with their digitalized evaluation in the discriminatory analysis of reactive atypia and dysplastic lesions in gastrointestinal biopsies of the stomach

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Abstract

The differential diagnosis between reactive atypia and non-invasive neoplasia (or dysplasia) can be challenging in the case of small conventional forceps biopsy specimens of the stomach. Despite the existence of several classifications for neoplastic epithelial lesions of the stomach, there are few auxiliary tools for aiding in this decision besides standard stains. We studied the utility of Ki-67 and p53 immunohistochemistry in this setting and their clinico-pathological correlations, based on a set of 99 cases with cytological or architectural atypia reviewed by three pathologists. We also tested a digitalized method based on the ImageJ software for the evaluation of Ki-67 expression to determine whether this could be of an additional help. **Conclusions:** Ki-67 and p53 expression correlates well with microscopic and morphological modifications in biopsies and can be a useful tool in confirming or dismissing an impression of dysplasia in routine pathological work-up. Digital processing is cumbersome and of limited value and it could only be of additional help if more automated methods are developed.

Keywords: reactive atypia, gastric dysplasia, gastric biopsy, Ki-67, p53, immunohistochemistry.

Introduction

The technical evolution and availability of endoscopy has led to an important increase of upper gastrointestinal (GI) tract biopsy material in the department of pathology. Upper GI endoscopies are performed for diagnostic, treatment, surveillance and screening purposes. From the large amount of biopsies sent to a department of pathology, a significant number present a challenge for discriminating between inflammatory, reactive, dysplastic (precancerous) and frankly invasive tumoral lesions. One of the delicate problems included within this spectrum represents the differential diagnosis between reactive epithelial atypia, low-grade dysplasia and high-grade dysplasia, which presents a significant intra- and inter-observer variability [1–3]. Reactive atypia is defined by the presence of cytological and architectural features of dysplastic epithelium or modifications that differ from normal but are not completely diagnostic for neoplasia. Whereas dysplasia represents cytological and architectural lesions that are unequivocally neoplastic but without evidence of invasive growth [4].

The importance of the topic is also reflected by the existence of multiple classification systems of gastric epithelial neoplasia, like the Japanese, Padova or Vienna

classifications [5–7]. Much accent has been put on differentiating between high-grade non-invasive intra-epithelial neoplasia and invasive carcinoma, indeed with a more serious clinical importance, but the importance of discriminating between reactive atypia and incipient low-grade neoplasia cannot be dismissed either, because of financial aspects and implications in clinical follow-up [8, 9].

Ki-67 and p53 expression is increased in areas of dysplasia compared with reactive and non-neoplastic epithelium, which can be demonstrated by immunohistochemical methods, and this can help confirm a histological impression of neoplasia [10, 11]. Mutations of the p53 gene lead to the loss of its function as a tumor suppressor, this being an important step in tumor development [12–14].

Immunohistochemical stainings and simple quantification operations, like Ki-67 proliferative index count, can be performed with the help of different software like ImageJ and ImmunoRatio, a possibility that is more and more accessible in the laboratory due to the development and spreading of slide digitalizing technologies [15].

The aim of the study was to assess the expression of Ki-67 and p53 in challenging cases of bioptic material from the stomach, evaluate clinico-pathological correlations and

the utility of these two markers in clinical practice, and explore a digitalized assessment method of these slides, having in mind a possible automated method for the future.

✎ Materials and Methods

We performed a retrospective study of biopsy specimens obtained through upper GI endoscopy and conventional forceps biopsy and received during a period of one year, between 2015 and 2016, at the Department of Pathology, Mureș County Hospital, Tîrgu Mureș, Romania. We examined a number of 99 cases presenting cytological or architectural atypia at the level of foveolar, glandular or metaplastic epithelium. Obviously non-neoplastic (inflammatory) lesions without significant morphological changes of the epithelium and cases with evidence of obvious invasion and desmoplasia were excluded.

The tissue samples had been prepared through fixation in 4% buffered formalin, embedding in paraffin and sectioning of the obtained paraffin block at 3–4 μ m thickness. Sections were stained with Hematoxylin and Eosin (HE) standard, and Periodic Acid Schiff (PAS)–Alcian blue special staining.

Immunohistochemistry was performed with Ki-67 clone MIB-1 from DAKO, with a 1:100 dilution of the primary antibody and with p53 clone DO-7 from LabVision and a dilution of 1:50. Slides were prepared manually with pre-treating through boiling in sodium citrate buffer (pH 10) for 60 minutes, at 90°C, and 3,3'-diaminobenzidine (DAB) visualization.

HE-stained slides combined with PAS–Alcian blue-stained sections were reviewed by three pathologists (ST, BJR and OSC) and cases were included in one of three categories using a modified variant of the Vienna classification of gastric epithelial neoplasia, according to the following: negative for dysplasia, low-grade intraepithelial neoplastic lesions (including indefinite for dysplasia and low-grade dysplasia) and high-grade intraepithelial neoplastic lesions. Clinical and pathological modifications were noted and quantified. These changes included nuclear enlargement, hyperchromasia, nuclear stratification, nuclear crowding, increased mitotic activity, nuclear pleomorphism and irregularity, loss of nuclear polarity, mucus depletion, glandular crowding, budding and branching. Beside these aspects, we assessed the presence and amount of inflammatory infiltrate (quantified as mild or marked), activity (defined by the presence of polymorphonuclear neutrophils), presence or absence and type of intestinal metaplasia (complete, incomplete or compound), and endoscopic aspect of the lesions (ulcerated, flat or elevated).

After determining the type of the lesions on HE-stained slides, immunohistochemical stainings with Ki-67 and p53 were examined. Ki-67 staining was assessed by determining the density of positive nuclei, expansion of proliferative area and presence of positive nuclei on the surface of the mucosa (surface maturation). P53 expression was assessed using a 3-point scoring system, according to the following: 0–10% of positive nuclei – score 1+; 10–50% of positive nuclei – score 2+; >50% of positive nuclei – score 3+.

For digital evaluation, a number of 30 cases were randomly selected to obtain 10 cases for every type of

lesion (reactive, low-grade dysplasia, high-grade dysplasia). The selected slides were photographed with a Nikon 800 microscope, with a 200 \times magnification, for every case the same sized surface area from the upper part of the mucosal biopsies. For digital analysis, we used the cell counter function of the ImageJ free software, in which we determined the Ki-67 proliferative index by taking in account the absolute number of Ki-67 positive epithelial cells.

Statistical assessment was done using the Microsoft Excel tables and the GraphPad InStat 3 statistical software (free access). We calculated the mean and standard deviation values (\pm SD). A *p*-value <0.05 with 95% confidence interval (CI) was considered statistically significant. We also turned to correlations, *chi*-square test, using frequency tables.

✎ Results

From the 99 cases examined with cytological or architectural atypia, 69 (69.69%) were classified as reactive and inflammatory lesions, 18 (18.18%) as mild dysplasia, and 12 (12.12%) cases as severe dysplasia.

Biopsies were from 53 men with mean age of 62.62 \pm 11.98 years, ranging between 36 and 86 years, and 46 females with mean age of 63.06 \pm 11.41 years, ranging between 37 and 82. Regarding age distribution, the difference between genders was not statistically significant (*p*=0.85).

The majority of lesions showing cytological or architectural atypia came from biopsies taken from macroscopically flat mucosa (total of 62.62%), followed by polypoid lesions (30.3%) and the least frequent ulcerated lesions (7.07%). Mild dysplasia was found in flat mucosa and polyps (total of 17 cases), and severe dysplasia manifested most frequently as an exophytic mass (8.08%).

Intestinal metaplasia was present in every category, with type II and III (incomplete intestinal metaplasia) being invariably present in every case with dysplasia associated with intestinal metaplasia (18 cases).

In most cases, inflammation was mild, with no marked amount of inflammatory infiltrate in any case of mild dysplasia, but with active inflammation present throughout the spectrum of the categories, being almost always present in the case of severe dysplasia (11 out of 12 cases).

The microscopic and pathological features of studied lesions are summarized in Table 1 and some of the lesions presented in Figure 1.

The association between clinico-pathological factors studied (amount of inflammation, intestinal metaplasia, active inflammation, endoscopic appearance) was statistically significant.

The evaluation of morphological variations evidenced on Ki-67 immunohistochemistry shows that expansion of the proliferative area was present in a significant amount of reactive lesions (25 out of 69 cases), with a high density of positive nuclei being present in 15 cases. In the setting of dysplasia expansion of the proliferative area, increase in the density of positively staining nuclei and the absence of surface maturation was consistent.

Modifications observed on Ki-67 immunohistochemistry are summarized in Table 2 and some of the lesions presented in Figure 2.

Table 1 – The clinico-pathological features of studied lesions

<i>n</i> =99	Reactive gastric lesions	Mild dysplasia	Severe dysplasia	<i>p</i>
Mild inflammatory infiltrate	61 (61.61%)	18 (18.18%)	5 (5.05%)	0.0001
Marked inflammatory infiltrate	8 (8.08%)	0	7 (7.07%)	
Without intestinal metaplasia	49 (49.49%)	7 (7.07%)	6 (6.06%)	0.03
Incomplete intestinal metaplasia	4 (4.04%)	1 (1.01%)	2 (2.02%)	
Complete intestinal metaplasia	4 (4.04%)	0	1 (1.01%)	
Composite intestinal metaplasia	12 (12.12%)	10 (10.1%)	3 (3.03%)	0.004
Without activity	41 (41.41%)	10 (10.1%)	1 (1.01%)	
Activity	28 (28.28%)	8 (8.08%)	11 (11.11%)	
Flat lesions	46 (46.46%)	13 (13.13%)	3 (3.03%)	
Polypoid lesions	17 (17.17%)	5 (5.05%)	8 (8.08%)	0.02
Ulcerated lesions	6 (6.06%)	0	1 (1.01%)	

Table 2 – Ki-67 expression

	Expansion of the proliferative area		Density of positive nuclear staining		Surface maturation	
	No	Yes	Low	High	Absent	Present
Reactive lesions	44	25	54	15	1	68
Low-grade dysplasia	5	13	5	13	10	8
High-grade dysplasia	0	12	1	11	12	0

Median value of the Ki-67 proliferative index as determined by semi-automated digitalized cell count was 100.6 ± 46.72 , with values between 55–196 for reactive and inflammatory lesions. Low-grade dysplasia showed a medium cell count of 175 ± 29.32 , ranging between 137–229. Median value for high-grade dysplasia was 221.81 ± 45.89 , with counts ranging between 167–298. The differences between the three categories were statistically significant: *p* – reactive *versus* low-grade dysplasia 0.0005; *p* – reactive *versus* high-grade dysplasia 0.0001; *p* – low-grade dysplasia *versus* high-grade dysplasia 0.01. Despite the median values of the three categories being different and statistically significant, there are considerable overlap, the lowest score for high-grade dysplasia being below the highest score obtained in the case of reactive lesions.

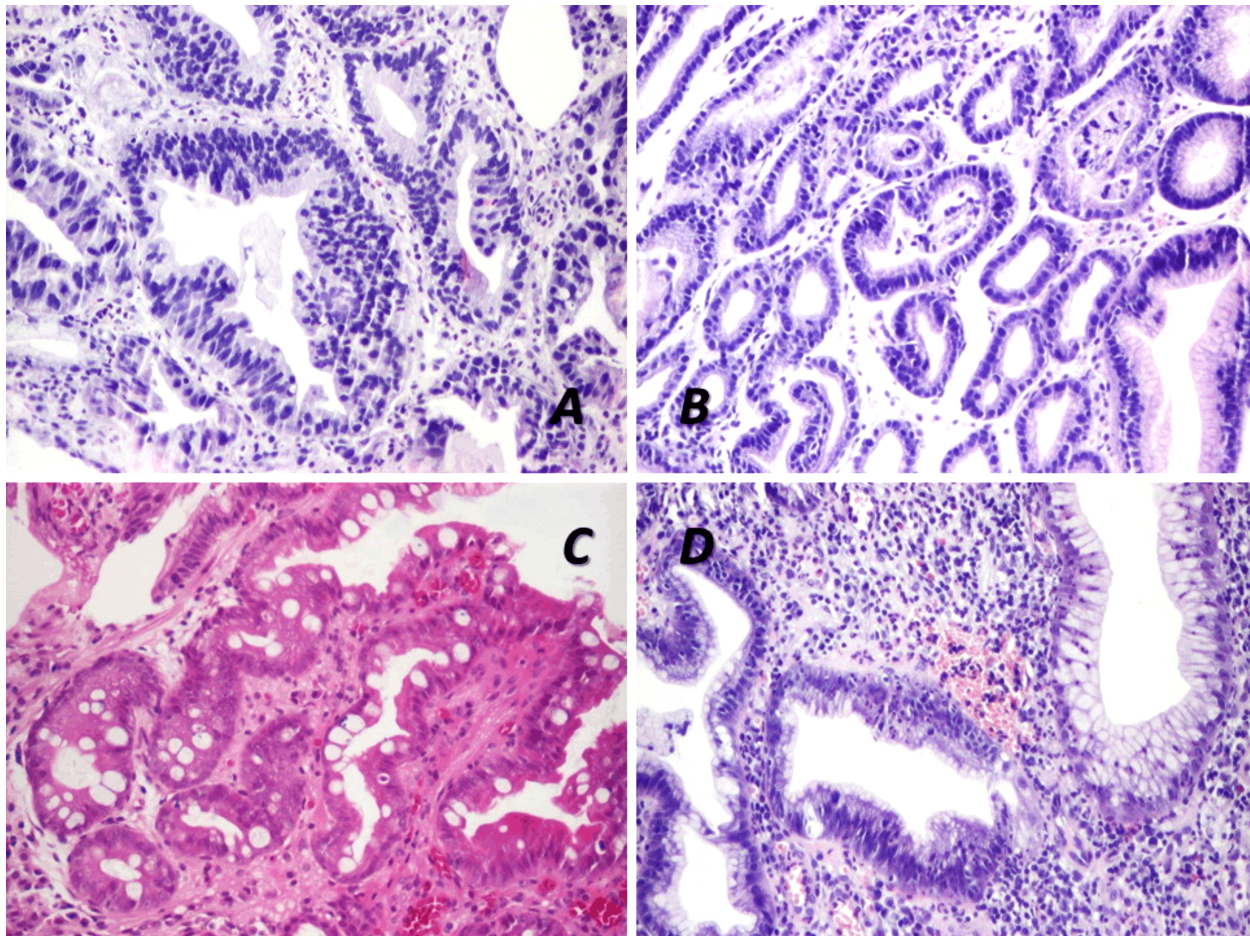


Figure 1 – (A) High-grade dysplasia; (B) Low-grade dysplasia; (C) Intestinal metaplasia with enlarged nuclei; (D) Reactive atypia with active inflammation. HE staining: (A) $\times 200$; (B–D) $\times 100$.

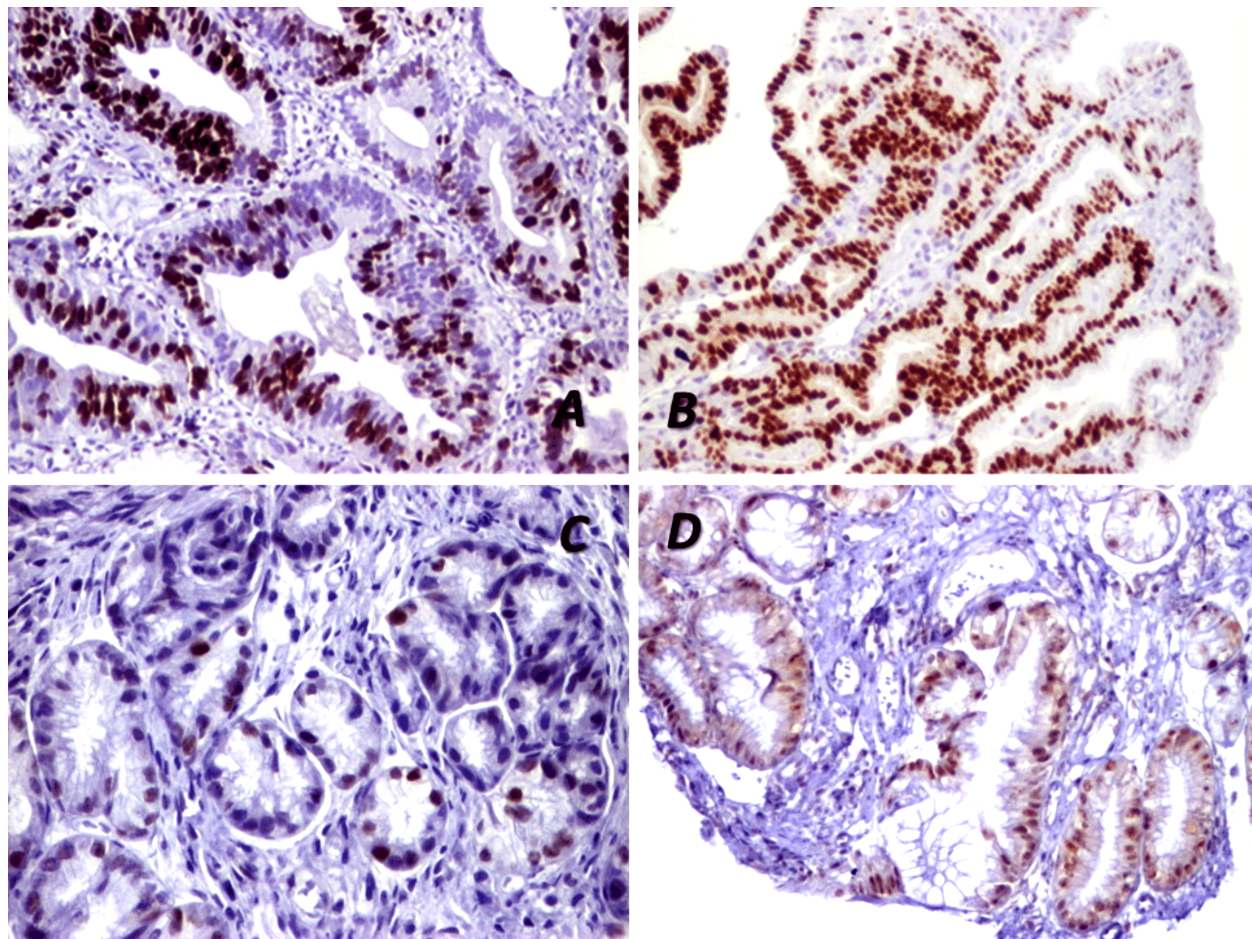


Figure 2 – (A) High-grade dysplasia, Ki-67 immunoreaction, $\times 200$; (B) Low-grade dysplasia, Ki-67 immunoreaction, $\times 100$; (C) Reactive atypia, p53 immunoreaction 1+, $\times 100$; (D) Reactive atypia, p53 immunoreaction 2+, $\times 100$.

The expression of p53 was as follows: in reactive-inflammatory lesions we found seven cases with 1+ score, and two cases presented a score of 2+. From the cases with low-grade dysplasia, three cases presented with a score of 1+, five cases a score of 2+, and two cases a score of 3+. High-grade dysplasia cases presented a score of 1+ in one case, a score of 2+ in five cases, and a score of 3+ in four cases.

We found a statistically significant correlation between the expression of p53 and the Ki-67 proliferation index in all of the three categories: reactive gastric lesions ($p=0.0094$), low-grade dysplasia ($p=0.0002$), and high-grade dysplasia ($p=0.0072$).

Discussion

One of the aims of the study was to evaluate the transition between reactive epithelial changes and dysplasia, and study the correlation of the Ki-67 and p53 expression with morphological criteria and clinical aspects. We based this evaluation on a slightly modified version of the Vienna classification of dysplasia, in that we included the indefinite for dysplasia category in the low-grade dysplasias. The rationale for this is that we consider most of these cases to be an incipient stage of low-grade dysplasia, and as such would benefit from the same clinical follow-up.

We found that gastric biopsies that presented atypia and needed differentiation between reactive and dysplastic

lesion came most frequently from flat mucosa (62%), followed by polypoid lesions (30%) and last by ulcerated or depressed lesions (7%), which was consistent with other studies [16].

Biopsies had been taken because of erosive gastritis, mucosal erythema, elevated or polypoid lesions, mucosal scars, ulcers, bile reflux and from gastric stumps, in the case of partial gastrectomy with gastro-enterostomy.

One of the most frequent cases with differential diagnostic issues was in biopsies obtained in the clinical setting of reactive gastropathy due to duodeno-gastric bile reflux, especially in specimens from the gastric stump of patients with gastric resection. Biopsies presented with significant cytological atypia, like nuclear enlargement, hyperchromasia, mucus depletion and nuclear irregularity. Crowded glands due to decreased intervening stroma or fibrosis give an aspect of architectural atypia. In these cases, the regenerative process produces an increase in activity of the proliferative compartment even in the superficial part of the mucosa. Immunohistochemical staining for Ki-67 reveals a much-expanded proliferative compartment, with increased density of positive nuclei. Clues for reactive changes represent the presence of rare neutrophils, the sometimes vaguely evident superficial maturation of the epithelium highlighted by Ki-67, and p53 negativity [17, 18].

Other scenario with marked cytological atypia appears in the setting of severe *Helicobacter pylori* infection, when cytological atypia is marked but architectural

irregularities are less frequent, thou may be observed sometimes near ulcers. In these cases, the presence of marked active inflammatory infiltrate is of major help, but the presence of abundant polymorphous infiltrate is also present in some of the high grade and invasive lesions. In this situation, Ki-67 expression may be marked and dense, even showing some extension to the surface of the mucosa, but p53 is usually negative [19].

Hyperplastic polyps can also show marked reactive atypia, and the architectural complexity of the branching foveolae can be confusing. Intestinal metaplasia can also be present. Usually, in this situations, the presence of active inflammation combined with highlighting of surface maturation with Ki-67 is of major help and p53 is negative [20].

Intestinal metaplasia might be another source of dilemma, since nuclear enlargement is always present. When associated with focal glandular crowding (reduced inter-glandular stroma), nuclear elongation, slight nuclear crowding with nuclei confined to the basal part of the epithelium but with evidence of some maturation to the surface it is a clear case of indefinite for dysplasia. As stated earlier, we consider that this so-called gastric hyperproliferative immature lesion is a step in the development of low-grade dysplasia in the Correa cascade of gastric cancer development. In this idea, when changes indefinite for dysplasia are present follow-up similar to low-grade dysplasia should be considered [21]. The presence of type II and III intestinal metaplasia in every case of dysplasia associated with intestinal metaplasia also supports the concept that this type could be the pathway through which intestinal type neoplasia develops in the stomach [22]. Gastric foveolar type dysplasia shows similar features with regenerative foveolar hyperplasia and Ki-67 immunohistochemistry demonstrating the surface maturation is of major help [23].

In the correct interpretation of abnormal proliferative activity, it has a major importance the knowledge of the normal proliferative area, which in the case of gastric epithelium is located in the isthmus, the part between the foveolae and gastric glands [24]. Ki-67 index is a good marker for proliferative activity and can indicate the more intense proliferation that is comparable with the grade of the neoplastic lesion [25, 26]. Absence of surface maturation as showed by Ki-67 expression on the surface of the mucosa was most significantly correlated with the presence of dysplasia. Similarly to other authors, we found an increased expression of p53 in high-grade dysplastic lesions compared with low-grade dysplasia and especially with reactive atypia [27].

The absolute values of positive nuclei in a predefined area determined by semi-automated digital counting with the free ImageJ software revealed interesting but not surprising facts. Some areas of reactive lesions gave similar values to low-grade or even high-grade dysplasia. Therefore, carefully choosing the area of counting is of major importance and correlating the collected information with other modifications is essential. Thou there were statistically significant differences among the three categories, considerable overlap in individual values between categories makes its worth as a help in this differential questionable. Moreover, the method of determining this value was cumbersome, time and resource consuming.

Conclusions

Immunohistochemical demonstration of Ki-67 and p53 overexpression correlates well with cytological and architectural morphological atypia. The most valuable marker is provided by Ki-67 expression through highlighting of surface maturation. Ki-67 combined with expression of p53 represent an efficient auxiliary tool in the differential diagnosis of reactive and dysplastic lesions in gastric biopsies. Digital analysis of Ki-67 expression can be of additional help only if significant automation is added to the procedure, because its application is time consuming and the data collected is only usable when correlated with morphological changes.

Conflict of interests

The authors declare that they have no conflict of interests.

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