

MOLECULAR MARKERS OF MUCOSA HARBORING GASTRIC ADENOMAS

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ABSTRACT - Context - Gastric adenoma is a precursor lesion of the adenocarcinoma. **Objective** - To characterize gastric adenomas according to the mucin immunorepression and to evaluate the immunorepression of p53, p16^{ink4a}, BCL-2, cyclin D, Ki-67, in the adenoma and in the gastric mucosa harboring adenoma. **Methods** - Forty gastric specimens from 20 patients were classified as intestinal (MUC2 - goblet cell mucin) or foveolar (MUC5AC - gastric-foveolar mucin) adenomas. Immunohistochemistry was performed using streptavidin-biotin-complex method. **Results** - Twelve (60%) patients were men. The mean age was 67.9 ± 12.9 years-old. Intestinal adenomas were detected in 13 (65%) patients and gastric type in 7 (35%). Low-grade dysplasia was present in 13 (65%) of the adenomas, high-grade in 3 (15%), and adenocarcinoma within the polyp in 4 (20%). Six (30%) patients had synchronous adenocarcinoma. p53 immunorepression was observed in 6/20 (30%) of adenomas, and in 2/6 (33.3%) of synchronous tumors. There was an association between p53 immunorepression and intestinal type of adenoma/tumor, $P = 0.04$. There was no association between p16^{ink4a}, Bcl-2, cyclin D and Ki-67 and adenoma clinicopathological characteristics. **Conclusion** - Immunohistochemistry may be useful to classify the adenomas subtypes and may define the pathway of adenoma to carcinoma sequence.

KEYWORDS - Stomach neoplasms. Adenocarcinoma. Tumor markers, biological. Gene expression regulation, neoplastic. Immunohistochemistry.

INTRODUCTION

Gastric adenoma is considered a precursor lesion for the development of adenocarcinoma in the stomach^(1, 24). It is defined as raised lesion of dysplastic epithelium and can be present as plain, villous, pedunculated or depressed type^(10, 16, 18, 19).

The adenomas can contain focus of intestinal differentiation, with goblet cells and/or Paneth cells (intestinal type), or only dysplastic foveolar epithelium (gastric type). It has been demonstrated that almost all adenomas of the intestinal type grow in a mucosa with metaplasia and gastric atrophy, and about 40% have high grade dysplasia. In contrast, adenomas of the foveolar type tend to develop in apparently normal epithelium, in a non atrophic mucosa and rarely present high degree of dysplasia or synchronous adenocarcinoma⁽²⁾.

Immunohistochemical staining for mucins may be useful to differentiate adenomas of gastric or foveolar phenotype, which are derived from the foveolar gastric epithelium of those of intestinal phenotype, which are derived from the metaplastic epithelium^(5, 22). Additionally, gastric carcinomas immunorepressing mucins of

the foveolar type are associated with worst prognosis and greater malignant potential, when compared to those with mucins of the intestinal type^(22, 23).

The adenoma subtype characterization is relevant because it may guide the treatment management (endoscopic intervention or surgical resections). Moreover, the routes of carcinogenesis of the gastric adenomas are controversial and the profile of genetic alteration is not completely known.

Thus, the aim of this investigation was to classify the adenomas according to the mucin immunorepression: MUC2 (mucin of the goblet cell) and MUC5AC (mucin of the gastric cell or foveolar); and to associate the phenotypic pattern of the adenomas with the immunorepression of p53 (tumor suppressor gene), p16^{INK4a} (tumor suppressor gene), Bcl-2 (Proto-oncogene - apoptosis), cyclin D (cell cycle) and Ki-67 (MIB-1) (cellular proliferation) in the adenomas and in the mucosa harboring them.

METHODS

Forty specimens from 20 patients who underwent gastric resection or endoscopic mucosal resection

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for gastric adenoma were analyzed. The biological material and clinical data were obtained at the Endoscopy Unit and Gastrointestinal Surgery Division, of the Department of Gastroenterology, University of São Paulo Medical School and the immunohistochemical analysis was carried out at the Pathology Department of the same University. This study was approved by the institutional review board of the Hospital das Clínicas of the University of São Paulo Medical School. Formalin fixed specimens were 4 mm sectioned, and the morphologic data was defined through H-E staining. Adjacent mucosa, with at least 3 cm of distance from the adenoma, were collected for comparative purposes.

Immunohistochemistry

The immunohistochemical method used was the streptavidin-biotin-peroxidase complex method. Molecular markers evaluated were: MUC2 clone Ccp58 (dilution 1:100), MUC5AC clone CLH2 (dilution 1:400), p53 clone DO7 (dilution 1:100), p16^{INK4a} clone 16P04 (dilution 1:200), Bcl-2 clone MO887 (dilution 1:80), cyclin D clone DCS-6 (dilution 1:200), and Ki-67 clone MIB-1 (dilution 1:50).

All the sections were heated in a 10 mM citrate buffer pH 6.0, except MUC5AC. Immunoreactions were visualized with diaminobenzidine (DAB)⁽²⁰⁾.

Histologic sections of stomach and colon adenocarcinoma, previously known for expressing high levels of p53, p16^{INK4a}, Bcl-2, cyclin D and Ki-67, mucins MUC2 and MUC5AC, were used as positive controls.

The negative controls corresponded to the same histological sections of colonic and gastric adenocarcinoma with the omission of the primary antibody. Nuclear (p53, p16^{INK4a}) and cytoplasmic immunoreactivity (p16^{INK4a}, Bcl-2, cyclin D and mucins), were classified quantitatively, in a scale of 0 to 4, for the intensity and distribution (intensity: 0 = absence staining; 1 = hardly visible staining; 2 = easily visible, however weak; 3 = intense, however not as much as the control; 4 = so intense as the control; distribution: 0 = absence of coloration or 75%)^(6, 7, 20).

p53 immunoreactivity was considered positive with intensity above 3 and distribution 3 or 4; for p16^{INK4a} some authors consider cytoplasmic positivity for p16^{INK4a} and others, the nuclear one. In this research, p16^{INK4a}, was considered positive if intensity and distribution were graduated as 2, 3 or 4. For Bcl-2 and Cyclin D, both are cytoplasmic stainings, and were considered positive if 3 or 4 for intensity and distribution^(6, 7, 18, 29).

For Ki-67, the cells were characterized as positive even if stained weakly. The Ki-67 index was calculated as the percentage of positive cells (counting the cells with positive nuclei and dividing themselves for the total number of cells). A minimum of 1,000 cells were counted, in the hot spot areas, using 400X magnification of an optic microscope. The Ki-67 marker was classified as high index of cellular proliferation when the positivity was greater than 25% of the cells and low index when the cellular distribution was lesser than 25% of the positive cells. This was based on the normal stomach immunoreactivity for Ki-67⁽¹⁸⁾.

Statistical analysis

Student *t* test was utilized to compare the quantitative data, and Fisher's exact test was calculated to analyze qualitative data. Both tests were considered significant when the *P* value was less than <0.05. The statistical program used was SPSS for Windows, version 18.0 (SPSS Incorporation Philadelphia, Pennsylvania, USA).

RESULTS

The clinical pathological characteristics of the 20 adenoma patients are presented in Table 1.

TABLE 1. Clinical-pathological comparisons between intestinal and foveolar adenomas according to mucin immunoreexpression

		Intestinal (MUC-2)	Foveolar (MUC5AC)	<i>P</i> value
Mean Age		67.1 ± 8.7	69.4 ± 9.6	0.71 [^]
Gender	Male	8	4	0.97*
	Female	5	3	
Size	<2 cm	11	0	0.0004*
	>2 cm	2	7	
Adenoma location	Proximal	4	4	0.35*
	Distal	9	3	
Growth pattern	Flat	5	2	0.62*
	Polypoid	5	5	
Viena's Classification	III	10	3	0.007*
	IV.1	3	0	
	IV.4	0	4	
Cancer Association	Synchronic	4	2	0.006*
	In the adenoma	0	4	
p53 - adenomas	Absent	9	1	0.04*
	Negative	7	7	
p16 ^{INK4a} cytoplasmic	Positive	6	0	0.97*
	Negative	5	2	
p16 ^{INK4a} nuclear	Positive	8	5	0.98*
	Negative	4	2	
Cyclin D	Positive	9	5	0.98*
	Negative	3	1	
Bcl-2	Positive	10	6	0.99*
	Negative	2	1	
Ki-67	<25%	11	6	0.61*
	>25%	3	3	
		10	4	

[^] = Student *t* test; * = Fisher exact test

Twelve (60%) patients were men. The mean age was 67.9 years-old, varying from 43 and 84 years-old.

As a result of mucin immunoeexpression (MUC2 and MUC5AC), the adenomas were classified as: 13 (65%) of intestinal type and 7 (35%) of the gastric type (Figures 1 and 2).

In relationship to the gastric adenoma location, seven cases were located at the body, one at the cardia and 12 at the antrum.

The size of the lesions varied from 0.8 cm to 6 cm in diameter. Nine lesions were larger than 2 cm and 11 lesions equal or smaller than 2 cm in diameter. The phenotype distribution according to the size of the adenoma demonstrated that 11/13 (65%) lesions of the intestinal type were smaller than 2 cm, while 2/13 were larger than 2 cm; 7/7 (100%) of the gastric type were larger than 2 cm, $P = 0.0004$. (Table 1) There was no association between lesion location and phenotype of the adenomas.

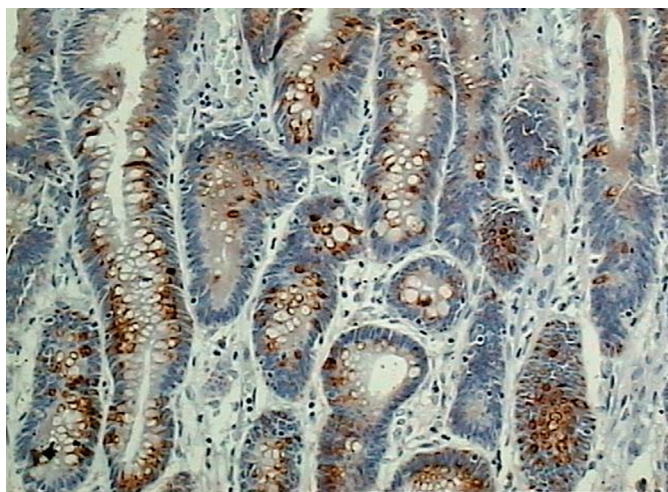


FIGURE 1. Intestinal type adenoma with mucin immunoeexpression for MUC2 (x20)

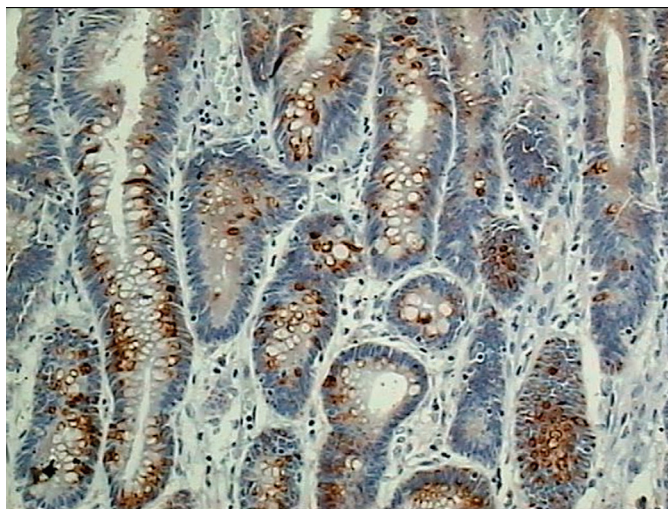


FIGURE 2. Gastric type adenoma with immunoreactivity for MUC5AC (x40)

The endoscopic findings were: six slightly elevated lesions, seven sessile and four elevated with central depression (Table 1). Such finding did not associate with mucin phenotype.

Accordingly to the modified Vienna's Classification, the intestinal type adenomas were classified as: 10/13 low grade (grade III.1 of Vienna) and 3/13 high grade (grade IV.1 of Vienna); and the gastric type adenomas, 3/7 were classified as low grade (grade III.1 of Vienna) and 4/7 were classified as intramucous carcinoma (grade IV.4 of Vienna) (Table 1).

Gastric adenocarcinoma were detected concurrently in 10/20 (50%) of the gastric adenomas cases. Of the 10 cases with cancer, 6 presented synchronous adenocarcinoma, corresponding to 30% (6/20) and 4 presented adenocarcinoma in the adenoma, corresponding to 20% (4/20) (Table 1).

In relationship to the adenomas with synchronous lesions, the mucin immunoeexpression (MUC2 and MUC5AC) demonstrated that in six adenomas cases with synchronous adenocarcinoma, two (33.3%) cases were classified as gastric type and four (66.7%) as intestinal type (Table 1).

All the patients had intestinal metaplasia and atrophy in the adjacent mucosa, independently of the histological type of the adenoma.

p53 adenoma immunoeexpression is presented in Table 1. Of the 20 adenomas, 6 presented positivity for p53 and all of them were intestinal type. While all positive p53 adenomas were classified as intestinal type, none adenoma of the gastric type were p53 positive. This difference was statistically significant ($P = 0.04$). Two/six (33%) positive synchronous adenocarcinoma for p53 were also of the intestinal type. On the other hand, p53 was negative in all four cases with focus of adenocarcinoma in the adenoma (Figures 3A and 3B).

Thirteen adenomas were positive for p16^{INK4a} considering the cytoplasm reactivity, and seven cases were negative. Considering the nuclear positivity, 14 cases of adenomas were positive and 6 cases were negative for the p16^{INK4a} (Table 1).



FIGURE 3A. p53 nuclear immunoeexpression in the adenoma is presented (x20)

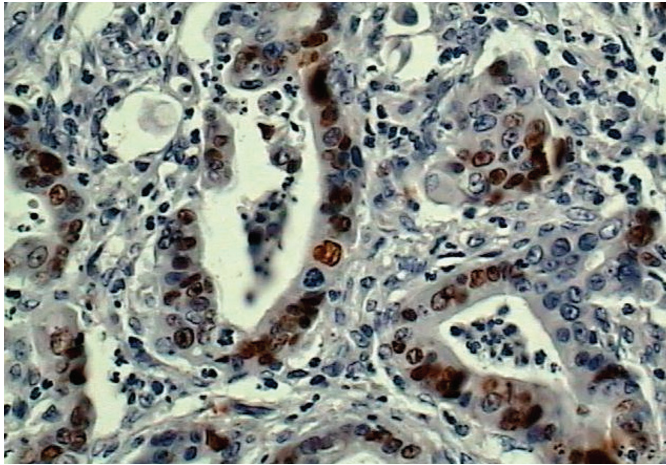


FIGURE 3B. p53 immunoreactivity in adenocarcinoma can be seen (x 40)

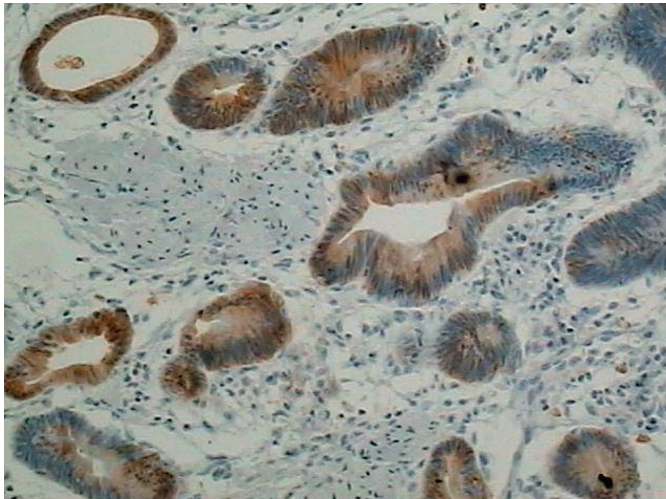


FIGURE 4A. Cytoplasmic immunopositivity for p16^{INK4a} in the adenoma (x20)

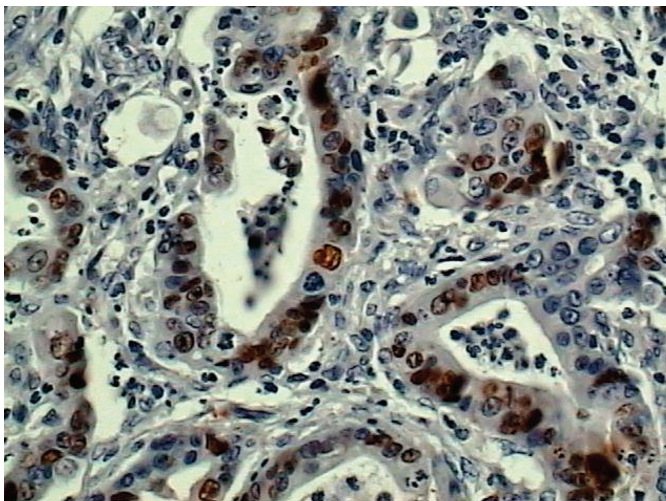


FIGURE 4B. Nuclear and cytoplasmic staining for p16^{INK4a} in the adenoma (x20)

Of the six analyzed cases of synchronous adenocarcinoma, four were positive for p16^{INK4a} and two were negative, considering both cytoplasmic and nuclear staining (Figures 4A and 4B).

Cyclin D immunopositivity was positive in 16/20 (80%) of the adenomas. In relationship to the cases of synchronous adenocarcinoma, 3/6 (50%) were positive for cyclin D (Figure 5).

Bcl-2 immunopositivity was noted in 17/20 (85%) cases of analyzed adenomas (Table 1). In the synchronous adenocarcinomas, four/six cases were positive for the Bcl-2 (Figure 6).

In the adenomas, 14/20 (70%) presented high index of cellular proliferation (higher than 25% of the cells) and 6/20 (30%) presented low index (lesser than 25%) (Table 1). There was no association between cellular proliferation and phenotype of adenoma or adenocarcinoma. High index of cellular proliferation was observed in 5/6 (83%) cases of synchronous

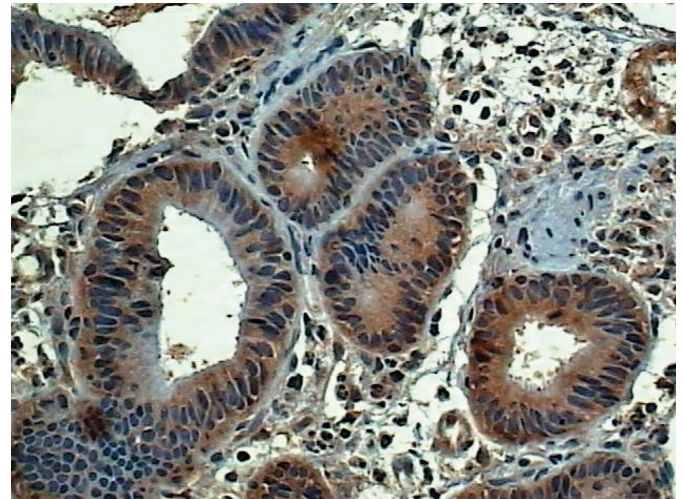


FIGURE 5. Cyclin D cytoplasmic immunopositivity was positive in the majority of the adenomas (x40)

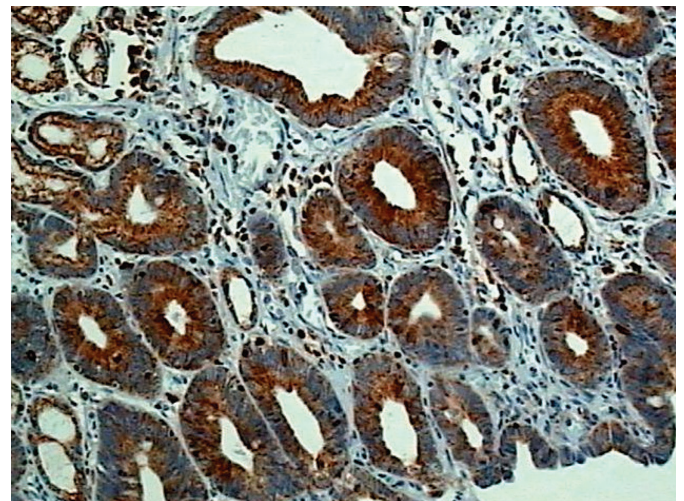


FIGURE 6. Bcl-2 cytoplasmic immunopositivity in the adenoma is noted (x20)

adenocarcinoma. In the synchronous adenocarcinoma, the cellular proliferation labeled index was higher than in the adenomas, $P = 0.049$. Figures 7A and 7B represent the result of immunohistochemistry for Ki-67 in the adenoma and adenocarcinoma, respectively (Figures 7A and 7B).

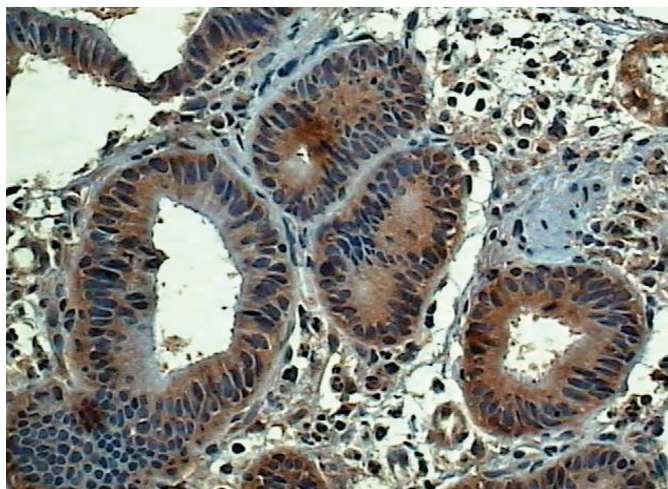


FIGURE 7A. Immunohistochemistry for Ki-67 in the adenoma (x10)

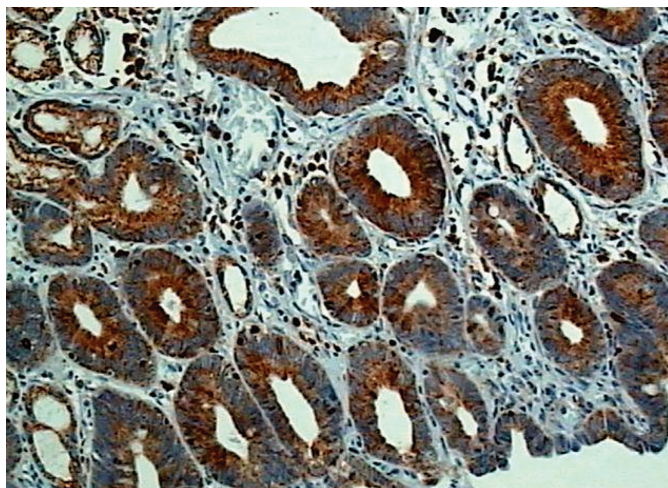


FIGURE 7B. Immunohistochemistry for Ki-67 in the adenocarcinoma (x20)

DISCUSSION

The incidence of gastric adenoma is low, varying from 0.1% to 2% of the patients⁽¹⁰⁾; however, the frequency of its malignant transformation is elevated, varying from 3.5% to 40%⁽⁴⁾. Nonetheless, numerous studies reported the association between gastric adenoma and carcinoma. The incidence of malignancy is dependent on the size of the adenoma. Adenomas smaller than 20 mm of diameter have 2%-10% of chance to harbor malignancy, while in those larger than 20 mm, these numbers go up to 40%-50%. Foci of adenocarcinoma were diagnosed in more than 80% of the adenomas larger than 4 cm⁽²⁵⁾.

Recently, the mucins have been utilized as a new diagnostic modality to explore the biological mechanism in the growth cellular lines and tumoral differentiation^(19, 22).

MUC 2 is a glycoprotein that is present in epithelial tissue, and its is associated with intestinal phenotype, being observed in the small intestine and colon and in the goblet cells of the intestinal metaplasia. It is not detected in normal gastric mucosa^(13, 15, 19, 23, 26).

MUC5AC is expressed in the surface of normal gastric mucosa cells, in the foveolar epithelium of the body and antrum. Its expression is associated to the gastric phenotype and is generally observed in gastric carcinomas of the diffuse type^(13, 15, 26).

In the present study, according to the mucin immunorexpression, there was a predominance of the intestinal type adenoma 13/20, and most of them were classified as type III according to modified Vienna's Classification (10/13), while most of the foveolar type adenoma were classified as type IV.4 (4/7). This data may indicate a more aggressive behavior for the foveolar type adenoma. Some authors also have reported higher frequency of intestinal type gastric adenomas based on the mucin immunorexpressions. These authors suggested that the tumoral tissue is a reflex of the phenotype of the adjacent mucosa. They concluded that the depressed adenoma and its adjacent mucosa with incomplete intestinal type phenotype presents neoplastic potential and must be followed carefully^(2, 19).

The clinical and pathological findings of the gastric adenomas are still not completed understood. In the present study, the five studied immunohistochemical markers were utilized to evaluate: proliferation and cellular control, Ki-67; gene Bcl-2 that participates of the programmed death cellular (apoptosis), tumor suppressor genes p53 and p16^{INK4a}, and gene that regulates the cellular cycle, as cyclin D.

The tumor suppressor gene p53 is located in the chromosome 17p, codifies a nuclear protein of 53 kD and is often involved in human carcinogenesis^(8, 20). It participates on the regulation of the cellular cycle, causing an inhibitory effect in the proliferation and transformation, causing the cell to rest in the G1 phase of the cellular cycle⁽¹⁴⁾. It is also responsible for the synthesis and repair of the DNA, being considered as the "genoma guardian". The loss of the function of p53 can result in defect in the replication of the DNA and malignant transformation⁽¹⁷⁾ with increase of the genomic instability, changes in ploidy and survival of the cells with increase number of mutations⁽¹⁴⁾.

p53 alteration has been demonstrated in up to 37.5% of the cases with intestinal metaplasia, from 30% to 60% in gastric adenomas or dysplasia and from 43% to 70% gastric carcinomas, indicating that p53 participates of the initial phases of gastric carcinogenesis^(20, 24).

In the present immunohistochemical study, p53 positivity in adenoma was demonstrated in 6/13 cases of intestinal type, while p53 was negative in all foveolar adenoma phenotype cases. Similarly, in synchronous adenocarcinoma, two/six positive cases for p53 also presented intestinal phenotype. Likewise, Sakurai et al.⁽²¹⁾ found p53 positivity in 12 of 32

adenomas (38%). Oya et al.⁽⁶⁾ also studied 50 cases of adenoma, correlating phenotype with the expression of p53. p53 immunopositivity was positive in the intestinal type adenomas and the indeterminate type, not being positive in the foveolar adenomas. Such finding strengthens the idea of distinct routes of carcinogenesis, one involving alterations of the p53 tumor suppressor gene as a foremost alteration.

The p16^{INK4a} gene is located in the chromosome 9p21 and is involved with the regulation of the cellular cycle⁽²⁸⁾. The p16^{INK4a} gene is inactivated by mutations, homozygous deletions, or methylation in diverse tumors of different origins and these methylation can result in deregulation of the cellular cycle⁽²⁹⁾.

In the present study, p16^{INK4a} cytoplasmic positivity was similar to the nuclear staining. In the synchronous adenocarcinomas, cytoplasmic and nuclear staining were observed in four/six cases, however there was no association with clinical and pathological data.

Bcl-2 altered immunopositivity has been detected in gastric premalignant lesions⁽⁶⁾. In this study, it was demonstrated high percentage of Bcl-2 positivity in the adenomas, (17/20 positives) and in adenocarcinomas (4/6 positives). However, there was no association of these findings with clinical and pathological characteristics of the lesions. Other authors have found decreased immunopositivity in the adenocarcinomas associated to the adenomas⁽²⁷⁾.

The cyclin D gene, which is identified in *prad-1* oncogene, codifies a protein that regulates the cellular cycle and is expressed in high levels during the G1 phase of the cellular cycle^(18,24). Although highly frequent, there was no association between Cyclin D immunopositivity in the adenomas or tumors, and clinicopathological characteristics.

Ki-67 antibody detects a nuclear protein of 345 to 395 kD, codified by a gene in the chromosome 10, which is present in G1, S, G2 and M phases of the cellular cycle, however absent in the cells in rest or G0²⁹. Cattoretti et al.⁽³⁾ produced MIB-1 monoclonal antibody, characterized as equivalent to the Ki-67, presenting the great advantage to be used in paraffin-embedded tissue blocks.

There was no difference in proliferation between the foveolar or intestinal phenotype, indicating that cell pro-

liferation is deregulated in both adenoma types, despite of mucin phenotype.

Immunohistochemical analysis can, therefore, be useful to better understand the clinical and pathological characteristics of the adenomas (intra-epithelial neoplasia). In the patients with intestinal type adenoma, in different locations of the stomach, a careful endoscopic investigation should be carried out, with multiple biopsies, since all the mucosa can be altered with the presence of intestinal metaplasia and atrophy and, consequently, with higher possibility of an association with another focus of adenocarcinoma. On the other side, adenomas of the foveolar type seem to be more aggressive lesions, with large size, and may require more extended local resection (endoscopic or surgical) to avoid local recurrence^(16,25).

CONCLUSION

According to the present study, it can be concluded that the mucin phenotype may be used to differentiate gastric adenomas (foveolar and intestinal type). There was a higher labeled index of proliferation (Ki-67) in carcinomas compared to adenomas, however, both adenoma types were highly proliferative compared to the normal mucosa. p53 overexpression was associated to the intestinal type adenoma and synchronous adenocarcinoma, indicating the same route of carcinogenesis. On the other hand, there was no association between the molecular markers p16^{INK4a}, cyclin D, Bcl-2 and the clinical and pathological characteristics of the mucosa harboring the adenomas. Therefore, immunohistochemistry panel of markers may be utilized to better understand the clinicopathological characteristics of the adenoma in the setting of mucosal damage and probably might be used to guide the appropriate treatment.

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RESUMO - Contexto - Adenoma gástrico é uma lesão precursora do adenocarcinoma. **Objetivo** - Melhor caracterizar os adenomas de acordo com a imunopositividade de mucinas e avaliar a imunopositividade de p53, p16^{INK4a}, BCL-2, cyclin D, Ki-67, nos adenomas e na mucosa gástrica adjacente.

Métodos - Quarenta espécimes gástricos provenientes de 20 pacientes portadores de adenomas foram classificados como do tipo intestinal (MUC2 – mucina presente nas células caliciformes) ou gástrico (MUC5AC – mucinas de padrão foveolar). Realizou-se imunohistoquímica para p53, p16^{INK4a}, BCL-2, cyclin D e Ki-67 pelo método do complexo da estreptavidina-biotina. **Resultados** - Doze (60%) pacientes eram homens e a média de idade foi de 67,9 ± 12,9 anos. Os adenomas foram classificados como do tipo intestinal em 13 (65%) pacientes e do tipo gástrico em 7 (35%). Displasia (neoplasia intraepitelial) de baixo grau estava presente em 13 (65%), displasia de alto grau em 3 (15%), e adenocarcinoma no pólipos adenomatoso em 4 (20%) pacientes. Observou-se imunopositividade do p53 em 6/20 (30%) adenomas, e em 2/6 (33,3%) dos tumores sincrônicos. Houve associação entre imunopositividade do p53 e adenoma/tumor tipo intestinal, *P* = 0,04. Não houve associação entre imunopositividade do p16^{INK4a}, Bcl-2, ciclina D e Ki-67 e as características clinicopatológicas dos adenomas. **Conclusão** - Imunohistoquímica pode ser utilizada para caracterizar os subtipos de adenoma e talvez indicar o caminho de carcinogênese.

DESCRIPTORES – Neoplasias do estômago. Adenocarcinoma. Marcadores biológicos de tumor. Regulação bacteriana da expressão gênica. Imunohistoquímica.

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