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p53 AND Ki-67 IN BARRETT'S CARCINOMA – is there any value to predict recurrence after circumferential endoscopic mucosal resection?

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ABSTRACT – ***Background*** - There are situations in which the specimens obtained after endoscopic mucosal resection of superficial adenocarcinoma arising from Barrett's esophagus are not adequate for histopathological assessment of the margins. In these cases, immunohistochemistry might be an useful tool for predicting cancer recurrence. ***Aims*** - To evaluate the value of p53 and Ki-67 immunohistochemistry in predicting the cancer recurrence in patients with Barrett's esophagus-related cancer referred to circumferential endoscopic mucosal resection. ***Methods*** - Mucosectomy specimens from 41 patients were analyzed. All endoscopic biopsies prior to endoscopic mucosal resection presented high-grade dysplasia and cancer was detected in 23 of them. Positive reactions were considered the intense coloration in the nuclei of at least 90% of the cells in each high-power magnification field, and immunostaining could be classified as superficial or diffuse according to the mucosal distribution of the stained nuclei. ***Results*** - Endoscopic mucosal resection samples detected cancer in 21 cases. In these cases, p53 immunohistochemistry revealed a diffuse positivity for the great majority of these cancers (90.5% vs. 20%), and Ki-67 showed a diffuse pattern for all cases (100% vs. 30%); conversely, patients without cancer revealed a superficial or negative pattern for p53 (80% vs. 9.5%) and Ki-67 (70% vs. 0%). During a mean follow-up of 31.6 months, 5 (12.2%) patients developed six episodes of recurrent cancer. Endoscopic mucosal resection specimens did not show any significant difference in the p53 and Ki-67 expression for patients developing cancer after endoscopic treatment. ***Conclusions*** - p53 and Ki-67 immunohistochemistry were useful to confirm the cancer; however, they had not value for predicting the recurrent carcinoma after circumferential endoscopic mucosal resection of Barrett's carcinoma.

HEADINGS – Barrett esophagus. Esophageal neoplasms. Adenocarcinoma. Ki-67 antigen. Tumor suppressor protein p53. Immunohistochemistry.

INTRODUCTION

It is well known that endoscopic mucosal resection (EMR), in addition to improvimg the diagnostic yield and accuracy of neoplastic staging as compared to endoscopic biopsies, offers a good treatment for Barrett's cancer when the lesion is limited to the mucosa. Besides, EMR is the most useful way to predict the cancer recurrence, with its contribution in tumoral stage revealing a significant impact on patient outcome[3, 17]. Unfortunately, there are situations where the specimens obtained from EMR are not adequate for the standard histopathological assessment, especially in the presence of small fragments. This way, tumoral markers might be an auxiliary tool for predicting local recurrence or the development of metachronous lesions.

Among different markers, p53 and Ki-67 are options to select those cases in higher risk for cancer development. The p53 tumor suppressor protein works as a transcription factor that controls the expression of many genes in the regulation of the cell cycle. Inactivation of its gene can contribute to the development of Barrett's adenocarcinoma. Likewise, Ki-67 expression has been used to evaluate the increased cell proliferation, one of the first steps in the carcinogenesis. Both markers show high expression in high grade dysplasia and adenocarcinoma[8, 11, 13, 20, 21]. Nevertheless, to date there is not a consensus about the value of these markers to predict the neoplastic recurrence after endoscopic treatment.

This study was carried out to evaluate the clinical value of the p53 and Ki-67 immunohistochemistry to predict local recurrence or the development of metachronous lesions after circumferential endoscopic mucosal resection of Barrett's esophagus-related cancer.

METHODS

Between February 1999 and December 2005, 41 consecutive patients with histologically proven Barrett's esophagus were referred to circumferential endoscopic mucosal resection of Barrett's esophagus-related high-grade dysplasia or mucosal cancer (Table 1). During the

This work was performed in the Endoscopy Unit and Department of Biopathology from Paoli-Calmettes Institute, Marseille, France.
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first procedure, the index lesion and its contiguous metaplastic tissue were resected. One month later, the second half of Barrett's epithelium was resected. The criteria for inclusion were age over 18 years; presence of specialized columnar epithelium confirmed by hematoxylin-eosin before endoscopic therapy, detection of mucosal adenocarcinoma or, at least, multifocal high-grade dysplasia, as well as marked surgical risk (heart disease, respiratory insufficiency, cirrhosis or poor general health) or a refusal to surgery. The mean length of the Barrett's epithelium was 4.9 cm (range: 1-15 cm). Endoscopic biopsies had previously detected high-grade dysplasia in all cases, and mucosal carcinoma was found in 23 of these patients. Patients with cancer were submitted to an endosonographic staging with a 10 MHz-radial ultrasonic transducer (EG 36UR or FG36 X; Pentax, Hamburg, Germany) 10–15 days before the first session of EMR. Overall, based on the pre-treatment endoscopic ultrasound, cancer was classified as T0N0 in one patient and T1N0 in 20 cases. Two additional cases were classified, respectively, as T2N0 and T1N1.

TABLE 1. Characteristics of patients submitted to circumferential EMR of Barrett's esophagus-related high-grade dysplasia or early adenocarcinoma

Patients (n)	41
Sex (M / F)	35 / 6
Age (years)	65.8 +/- 10.5
Extension of BE (cm)	4.9 +/- 3.4
Size of visible lesions (mm)	14.4 +/- 7.6
Histological diagnosis prior to EMR	
High-grade dysplasia	18 (44%)
In situ adenocarcinoma	23 (56%)
Ultrasound tumoral stage #	
T0	1 (4.3%)
T1	21 (91.3%)
T2	1 (4.3%)
Ultrasound nodal Stage #	
N0	22 (95.7%)
N1	1 (4.3%)

Endoscopic ultrasound evaluation was performed prior to EMR only in whom endoscopic biopsies detected mucosal cancer

Histopathological assessment

The material consisted of esophageal mucosectomy specimens, which were fixed in 4% phosphate buffered formaldehyde, and embedded in paraffin blocks. All slides were stained with hematoxylin-eosin and reviewed by two experienced gastrointestinal pathologists (GM and HM). The samples were analysed according to the Paris classification[18] and classified as presence of metaplastic epithelium, negative for intraepithelial neoplasia, low grade intraepithelial neoplasia, high-grade neoplasia (either intraepithelial or intramucosal) or submucosal carcinoma. Lesions with high-grade intraepithelial neoplasia and no invasion of the lamina propria are called high-grade dysplasia, and intramucosal carcinoma in the presence of invasion of the lamina propria. Beyond this level, all neoplastic lesions with invasion of the submucosa are called invasive carcinoma. In the presence of intramucosal carcinoma, three additional subclasses are described: m1 (intraepithelial), m2 (the lamina propria is affected) and m3 (the *muscularis mucosae* is affected). The submucosal carcinoma is also divided into three categories: Sm1, Sm2 and Sm3 (the cutoff value to distinct between Sm1 and Sm2 categories is 500 μm).

Immunohistochemical staining

The immunohistochemistry expression of p53 and Ki-67 were assessed by the streptavidin-biotin peroxidase method on five-micrometer histological sections obtained from the tissue blocks that presented the most significant lesion, as detected by hematoxylin-eosin staining. The primary antibodies were mouse monoclonal antibody against human p53 protein (clone DO-7, Immunotech, Marseille, France) and rabbit monoclonal antibody against human Ki-67/SP6 (NeoMarker, Fremint, CA, USA) both diluted at 1:200. The chromogen used was diaminobenzidine. Immunohistochemical reactions for both markers were considered positive in the presence of intense brown coloration in the nuclei of at least 90% of the cells in each high-power magnification field (400x)[1, 7], and immunostaining pattern was classified as superficial (luminal glands from the upper half of the lamina propria) or diffuse (the entire width of the epithelium, from the top to the basal layer) according to the mucosal distribution of the stained nuclei. Positive control was breast carcinoma and negative control was a section stained as above but without the primary antibody.

Statistical analysis

The significance level was 5% for all statistical procedures. Numerical variables were expressed as mean ± standard deviation and comparative analysis between them were performed by Student *t* test. All categorical data were analysed by chi-square test with Yates correction and Fischer exact test.

This study was approved by the Research Ethics Board of our Institution and written informed consent was obtained from each patient before the endoscopic treatment.

RESULTS

During a mean follow-up of 31.6 (range:0-83) months, 41 patients were submitted to 63 sessions of EMR (mean number of sessions:1.5). Barrett's epithelium recurred in 10 (24.4%) patients, after a mean follow-up of 49.6 weeks (3-193 weeks). All these cases were submitted to a new EMR. From this same group, five patients (12.2%) developed recurrent (four cases) or metachronous carcinomas (two cases) after a mean follow-up of 34.5 (3-72) weeks. Barrett's metaplasia and cancer recurred twice in one patient. All these cases, except one patient referred to surgery, underwent a new EMR, and three of them received adjuvant therapy with argon plasma coagulation (two cases) or chemoradiotherapy (one case). Overall, in an intention-to-treat manner, 31 (75.6%) patients had the metaplastic epithelium completely replaced by the squamous epithelium until the conclusion of the study. Including those patients submitted to a new EMR after recurrence of metaplastic epithelium or carcinoma, the success rate of the endoscopic approach was 90% (37/41).

Barrett's specimens obtained from EMR revealed cancer in 21 cases. In these cases, the diagnoses obtained by endoscopic

forceps biopsies prior to mucosectomy were mucosal cancer (17 cases) and high-grade dysplasia (4 cases). The histopathology revealed submucosal involvement in eight patients (six cases Sm1 and two cases Sm2) previously diagnosed as mucosal cancers by pre-EMR biopsies. Besides, EMR confirmed the cancer in 17/23 (74%) cases whose pre-EMR biopsies had previously detected mucosal cancer. Two cases were diagnosed as high-grade dysplasia and four cases as low-grade dysplasia.

Specifically about the endoscopic ultrasound evaluation, 21 (91.3%) of 23 cases were classified as mucosal tumors, except for 1 patient diagnosed as a T2 cancer, in whom the EMR specimen revealed a T1Sm2 tumor, and another case classified as T1N1, in which EMR detected only low-grade dysplasia.

In relation to the immunohistochemistry, both markers were very useful in confirming those cases with cancer detected in EMR samples. The great majority of these cases revealed a diffuse positivity in relation to p53 (Figure 1), and Ki-67 showed a diffuse positivity in all cases. In these patients, there was a statistically significant difference in the staining patterns, revealing a diffuse positivity. On the other hand, in those cases without cancer, there was only a superficial positivity or no staining (Table 2).

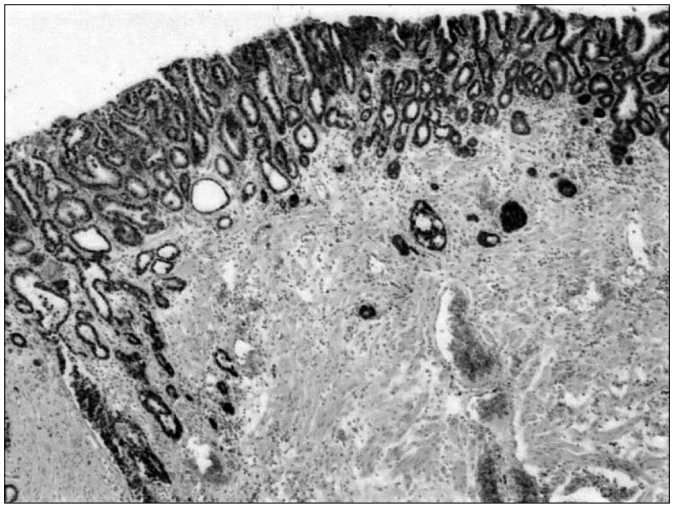

FIGURE 1. Barrett's cancer infiltrating into the deeper layers of the *muscularis mucosae*. There is no infiltration into the submucosa (Tism3). Diffuse p53 immunostaining (magnification: 100x)

TABLE 2. p53 and Ki-67 immunohistochemistry in Barrett's esophagus according to the presence of cancer in EMR specimens

	With cancer (n = 21)	Without cancer (n = 20)	*P*
p53 in EMR specimens			
Negative	0	8 (40%)	<0.05
Superficial positivity	2 (9.5%)	8 (40%)	NS
Diffuse positivity	19 (90.5%)	4 (20%)	<0.001
Ki-67 in EMR specimens			
Negative	0	3 (15%)	NS
Superficial positivity	0	11 (55%)	<0.001
Diffuse positivity	21 (100%)	6 (30%)	<0.001

NS = non significant (*P*>0.05)

Concerning the predictive value of p53 and Ki-67 immunohistochemistry for recurrent or metachronous carcinoma after EMR of Barrett's esophagus-related high-grade dysplasia or superficial adenocarcinoma, no statistical difference was detected (Table 3).

TABLE 3. Recurrence of neoplasia after EMR of Barrett's esophagus-related high-grade dysplasia or early adenocarcinoma according to p53 and Ki-67 status

	Recurrence (n = 6)	No recurrence (n = 35)	*P*
p53 in EMR specimens			
Negative	1 (16.7%)	7 (20%)	NS
Superficial positivity	1 (16.7%)	9 (25.7%)	NS
Diffuse positivity	4 (66.7%)	19 (54.3%)	NS
Ki-67 in EMR specimens			
Negative	1 (16.7%)	2 (5.7%)	NS
Superficial positivity	1 (16.7%)	10 (28.6%)	NS
Diffuse positivity	4 (66.7%)	23 (65.7%)	NS

NS: non significant (*P*>0.05)

DISCUSSION

In our experience, EMR offered improved diagnosis and staging as compared to endoscopic forceps biopsies and endoscopic ultrasound. MAY et al.[15] demonstrated that the sensitivity of endoscopic ultrasound for esophageal submucosal cancer was 48%, and the understaging for this same group was 52%. In the experience of MINO-KENUDSON et al.[17], endoscopic ultrasound correctly reported an intramucosal or submucosal lesion in 70% of the cases. The biopsy diagnosis corresponded to the EMR diagnosis in 63% of the cases, occurring understaging in 21% of the cases. These data are similar to those detected in our series, and they could explain our eight patients previously diagnosed as mucosal cancers, in whom only the mucosectomy specimens revealed the submucosal involvement.

The use of molecular biology in order to detect those cases of Barrett´s esophagus more prone to develop cancer, as well as to better assess the response to different therapeutic approaches have been emphasized in the literature[5, 19]. In this respect, the utilization of p53 and Ki-67 are widely applicable[2, 8, 9, 10, 11, 12, 13, 24], as both markers can be evaluated by immunohistochemistry, a relatively simple, fast, and cheap technique available in most pathology laboratories[16].

Nevertheless, there is no consensus on the positivity criteria for p53 and Ki-67 immunohistochemistry in the setting of Barrett's esophagus neoplasia. As there are still no uniform standards to define positivity for both markers, we chosed the presence of, at least, 90% of the nuclei stained in each field of high-power magnification to define positive immunostaining. Once positive, the staining pattern was classified as superficial or diffuse, according to the mucosal distribution of the stained nuclei. With these criteria, we precluded the major difficulties inherent to multiple grading scales, which could lead to important intra-and interobserver variability[4]. Specifically for the p53, with this criterion we also increase the likelihood of detecting mutations[1, 7]. Moreover, only the numerical aspect of p53 expression was considered in the quantification of its expression, with no intensity threshold

being considered, as suggested by HALL and LANE[7]. The occurrence of few cells with intense nuclear staining does not seem to correlate with molecular abnormalities of p53. In turn, the presence of intense nuclear staining in most cells is often associated with mutations[1, 7].

In our study, p53 and Ki-67 were detected in all cases with Barrett's cancer, revealing a diffuse pattern of staining for the great majority of these cases. Specifically for the p53 expression, there was a significant difference in relation to those cases without cancer and harboring only high grade dysplasia (P<0.001). Similarly, Ki-67 expression also showed a diffuse pattern in all cases with cancer (P<0.001). There were some cases with high grade dysplasia but no cancer expressing both markers, but p53 and Ki-67 showed a diffuse positivity solely in 20% and 30% of the cases, respectively. In fact, p53 was negative or presented only a superficial expression in 80% of these cases, and the same was true for Ki-67 in 70% of all patients with no cancer. So, in addition to confirm the cancer suspected by routine histopathological assessment, expression of p53 and Ki-67 can be valuable tools for the diagnosis of mucosal adenocarcinoma in suspicious cases, as previously demonstrated in the literature[11, 24].

Although a good auxiliary tool to the correct diagnosis of cancer in mucosectomy specimens, the value of the immunohistochemistry was somewhat disappointing for predicting the cancer recurrence after endoscopic treatment. Patients developing cancer recurrence more usually showed a diffuse positivity, accounting for almost 67% for both markers. However, the same was true for those cases without cancer recurrence, in whom a diffuse positivity for p53 and Ki-67 were detected in, respectively, 54% and 65% of the patients. Indeed, there was no statistically significant difference between these findings, which could be a bias due to the limited number of cases.

There are surgical series of invasive carcinomas evaluating these immunohistochemical markers with respect to the prognosis after surgical resection. There was no significant survival difference between patients p53 positive and those who were p53 negative[6, 9, 23]. Likewise, several papers have already been published analysing these tumor markers in the classic metaplasia-dysplasia-adenocarcinoma sequence[2, 5, 8, 19], although not evaluating the recurrence after any sort of endoscopic treatment.

This is the first study to evaluate the role of the immunohistochemistry in predicting cancer recurrence after total resection of Barrett's related high-grade dysplasia or early adenocarcinoma by means of the circumferential EMR[14, 22]. With the data obtained so far, p53 and Ki-67 immunohistochemistry in patients with endoscopically resectable disease submitted to circumferential EMR do not seem to be the ideal approach to evaluate the protective effect of the endoscopic resection against the cancer recurrence. Nonetheless, these results must be interpreted carefully, because we do not really know whether those patients expressing a diffuse positivity for one or both markers but with no cancer recurrence will develop or not a new adenocarcinoma in the future. Further clinical trials with longer follow-ups are necessary to clarify this question.

Indeed, it is well known that the histopathology of the mucosectomy specimens is the best manner to predict the recurrence, and it might be another reason to explain the lack of studies evaluating the immunohistochemistry. Our study highlights and reinforces that p53 and ki-67 immunohistochemistry is not useful to predict neoplastic recurrence after EMR. Technical improvement for en bloc resections and a better handling of the resected specimen are needed to save costs with unnecessary diagnostic tools, and to detect those high risk patients in whom this promising endoscopic treatment is not adequate for curative treatment, for whom either endoscopic surveillance or surgical resection is mandatory.

CONCLUSIONS

p53 and Ki-67 immunohistochemistry were useful to confirm the cancer arising from Barrett's esophagus. Nevertheless, both markers had not value for predicting the recurrent cancer after circumferential EMR of Barrett's esophagus-related carcinoma.

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RESUMO – ***Racional*** - Há situações nas quais o material obtido após mucosectomia endoscópica do adenocarcinoma superficial do esôfago de Barrett é inadequado para avaliação histopatológica de suas margens. Nesses casos, a imunoistoquímica poderia ser de auxílio para predição da recurrência tumoral. ***Objetivo*** - Avaliar o valor da detecção imunoistoquímica da p53 e do Ki-67 na predição da recurrência tumoral após mucosectomia endoscópica circunferencial do câncer no esôfago de Barrett. ***Métodos*** - Foi analisado o material proveniente de mucosectomias de 41 pacientes. Todas as biopsias endoscópicas pré-mucosectomia apresentavam displasia de alto grau e câncer foi detectado em 23 casos. A imunorreatividade foi definida pela coloração de, pelo menos, 90% dos núcleos em cada campo de grande aumento, podendo ser classificada como superficial ou difusa, conforme a distribuição celular dos núcleos corados. ***Resultados*** - A mucosectomia detectou o câncer em 21 casos. Nesses casos, a p53 revelou padrão difuso de positividade para a maioria dos casos (90,5% vs. 20%) e o Ki-67 demonstrou padrão difuso para todos os portadores de câncer (100% vs. 30%). Por sua vez, pacientes sem câncer revelaram padrão negativo ou apenas superficial para a p53 (80% vs. 9,5%) e para o Ki-67 (70% vs. 0%). Durante seguimento médio de 31,6 meses, cinco (12,2%) pacientes apresentaram seis episódios de câncer recurrente. Neste grupo, os fragmentos de mucosectomia não demonstraram nenhuma diferença significativa na expressão imunoistoquímica da p53 e do Ki-67 nos pacientes desenvolvendo câncer após o tratamento endoscópico. ***Conclusões*** - A imunoistoquímica da p53 e do Ki-67 é útil na confirmação do câncer; contudo não demonstra nenhum valor na predição da recurrência tumoral após mucosectomia endoscópica circunferencial do esôfago de Barrett com adenocarcinoma.

DESCRITORES – Esôfago de Barrett. Neoplasias esofágicas. Adenocarcinoma. Antígeno Ki-67. Proteína supressora de tumor p53. Imunoistoquímica.

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