

PROTEIN P16^{INK4a} IMMUNOHISTOCHEMICAL EXPRESSION IN ADENOCARCINOMA OF THE ESOPHAGUS

Avaliação da expressão imunoistoquímica da proteína p16^{INK4a} no adenocarcinoma de esôfago

Mário Henrique **OSANAI**, Maria Isabel Albano **EDELWEISS**, Luise **MEURER**, Maitê de Mello **VILWOCK**,
Leandro Totti **CAVAZZOLA**, Guilherme **RIBEIRO**, Carlos Cauduro **SCHIRMER**, Richard Ricachenevsky **GURSKI**

From Hospital de Clínicas de Porto Alegre and the Graduate Program in Medicine, Surgery, Faculty of Medicine, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

ABSTRACT - Background - The esophageal adenocarcinoma shows an increasing frequency in the last decades, specially in the developed countries. The Barrett's esophagus is accepted as the major premalignant lesion and the metaplasia-dysplasia-adenocarcinoma sequence presents a lot of genetic changes since its early events. The alterations in p16^{INK4a} are frequent in Barrett's esophagus and esophageal carcinoma. **Aim** - To verify the prevalence of the immunohistochemical expression of the p16^{INK4a} protein in patients with esophageal adenocarcinoma. **Methods** - The study population consisted of 37 patients with resected esophageal adenocarcinoma. The p16^{INK4a} protein expression was determined by immunohistochemistry using primary antibody p16^{INK4a}Ab-7, clone 16P07 NeoMarkers and assessed according to the Immunoreactive scoring system (IRS). **Results** - Of 37 analyzed patients, the most were male (86,5%) and the advanced disease was predominant (stages III and IV = 67,5%). In 12 (32,4%) the immunohistochemistry was positive for p16^{INK4a}. There was no significative relation between the protein expression and the degrees of histological differentiation of the biopsies and surgical specimens (p=0,81) neither with the staging (p=0,485). **Conclusion** - The lost of the immunohistochemical expression of the p16^{INK4a} protein in this study suggests that p16 is enroled in the carcinogenesis of the adenocarcinoma of esophagus.

HEADINGS - Esophagus. Esophageal neoplasm. Genes, p16.

Correspondence:

Richard Gurski,
e-mail: rgurski@iad.com.br

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RESUMO - Introdução - O adenocarcinoma de esôfago apresenta aumento de frequência nas últimas décadas, particularmente em países desenvolvidos. O esôfago de Barrett é reconhecido como a principal lesão precursora e o estudo da sequência metaplasia-displasia-adenocarcinoma mostra a ocorrência de alterações genéticas desde suas fases mais incipientes. As alterações no p16^{INK4a} são relatadas como frequentes no esôfago de Barrett e no carcinoma de esôfago. **Objetivo** - Verificar a prevalência da expressão imunoistoquímica da proteína p16^{INK4a} em exames anatomopatológicos de pacientes com adenocarcinoma de esôfago. **Método** - A população do estudo foi constituída de 37 pacientes com adenocarcinoma de esôfago. A expressão da proteína p16 foi detectada por meio de análise imunoistoquímica, com anticorpo primário p16^{INK4a}Ab-7, clone 16P07, NeoMarkers e avaliada de acordo com o Sistema de Escore de Imunoreatividade (Immunoreactive scoring system – IRS) modificado. **Resultados** - No grupo houve predominância de pacientes do sexo masculino (86,5%) e a maioria dos casos correspondia a estádios avançados (III e IV = 67,5%). Em 12 casos (32,4%) foi identificada expressão imunoistoquímica da proteína p16^{INK4a}. Não foi observada relação significativa entre a perda da expressão da proteína p16^{INK4a} e o grau de diferenciação histológica (p=0,81) nem com o estadiamento da doença (p=0,485). **Conclusão** - Ocorre perda da expressão imunoistoquímica da proteína p16^{INK4a}, corroborando as informações de que a inativação do gene p16 é um evento frequente e que pode exercer papel importante na carcinogênese do adenocarcinoma de esôfago.

DESCRITORES - Esôfago. Neoplasia de esôfago. Genes p16.

INTRODUCTION

Several studies have analyzed risk factors and mechanism of progression from metaplasia to dysplasia lesions of the adenocarcinoma^{14,15,22}. The risk of developing adenocarcinoma in patients with Barrett's esophagus (BE) is estimated at one case per 56 to 250 patient-years of follow-up¹⁰.

Changes of specific genes related to some types of tumors, show the important role of these genes as potential indicators of prognosis or response to therapy^{9,10}.

Changes in gene p16^{INK4a} (p16) are the object of study in several tumors such as squamous cell carcinoma of the head, neck and esophagus, adenocarcinoma of the pancreas and gastrointestinal stromal tumors^{10,24,26,32}. Studies in tumors of the pancreas and gastrointestinal stromal tumors reported worse prognosis in the presence of alterations in this gene^{10,24}.

There are many studies about the changes in p16 gene in Barrett's esophagus and esophageal carcinoma, including adenocarcinoma, and allelic loss of p16 gene alteration considered the initial progression of Barrett's metaplasia and hypermethylation, one of the most frequent inactivation of the gene^{4,16,28}. However, there are few publications focusing on the impact of these genetic and epigenetic changes in p16 protein expression in adenocarcinomas of the esophagus⁴.

Thus, this study aims to assess the prevalence of immunohistochemical expression of p16^{INK4a} protein in pathological examinations of patients with adenocarcinoma of the esophagus.

METHOD

This study was submitted and approved by regulatory and ethical guidelines committee of the institution, under protocol number 03-183.

The population studied was obtained by review of medical records of patients from Hospital de Clínicas de Porto Alegre, Brazil with adenocarcinoma of the esophagus.

The inclusion criteria was: patients with pathological diagnosis of adenocarcinoma of the esophagus. The exclusion criteria were: history or presence of other concomitant malignancy, neoadjuvant treatment (chemotherapy or radiation therapy), lack of paraffin blocks for the manufacture of blades for the study.

The variables studied were age, gender, stage, histological grade of differentiation, surgical procedure, the intensity of p16^{INK4a} protein expression, percentage of nuclei stained for p16^{INK4a}.

Cuts representative of the tumor to

immunohistochemistry were from paraffin blocks of biopsy material and surgical technique using standardize routine of the Pathology Department of Hospital de Clínicas de Porto Alegre. It consisted of deparaffinization and rehydration, antigen retrieval, inactivation of endogenous peroxidase and blocking of nonspecific reactions. The primary antibody, clone 16P07 Neomarkers p16^{INK4a} was incubated for 12 h at 4° C at a dilution of 1:75, followed by application of streptavidin-biotin complex-peroxidase (LSAB, Dako) and revelation with diaminobenzidine tetrahydroclorido Kit (DAB, Dako). The reaction presented as a positive control cervical intraepithelial neoplasia positive for p16, and the negative control performed without the use of primary antibody.

The evaluation of p16^{INK4a} (p16) was performed according to the scoring system of immunoreactivity (immunoreactive Scoring System - IRS)²¹ modified and is considered as positive only nuclear staining.

Two staining patterns were: positive - when at least 10% of nuclei were positive in patchy or diffuse through the tissue -, and negative when none or few cells (<10%) showed nuclear staining.

The staining intensity was classified graduating from 0 to 3, where 0 is negative, 1 is weak, 2 moderate and 3 is strong.

The final score of the expression of p16 was obtained by multiplying the two scores.

The tissue was analysed by two pathologists, independently and without knowledge of clinicopathological data. The final result was obtained by review of consensus between the two pathologists.

In Figure 1 can be seen, in several examples of staining, the intensities to better illustrate the method.

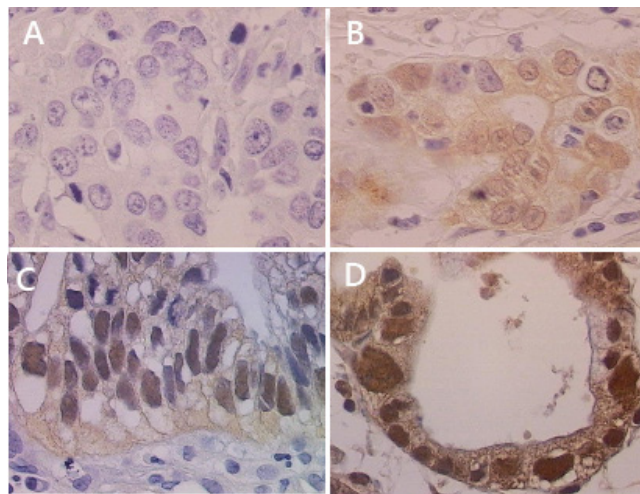


FIGURE 1 - Immunohistochemical expression of p16 (400X): A) negative; B) weak; C) moderate and D) strong

In statistical analysis, quantitative data are presented as mean and standard deviation, and categorical by frequency (absolute number) and percentage. The observations, made by two pathologists responsible for assessing the intensity of staining and p16 expression of their agreement, were estimated by Kappa measure of agreement. The distribution of p16 between groups and between histologic stages of disease was compared by the chi-square method. The data were processed and analyzed using SPSS (Statistical Package for Social Sciences) version 13.0.

RESULTS

Were reviewed medical records of 63 patients who met the inclusion criteria. Were included in the study only those who had paraffin blocks with biopsies or surgical specimens compatible for study, forming a population of 37 patients.

The average age was 61.9 years (standard deviation \pm 8.6 years). Thirty-two patients were male (86.5%), with a mean age of 61.2 years (SD \pm 1.6 years) and five were female (13.5%), mean age of 66 years (SD \pm 1.1 years) (Figure 2).

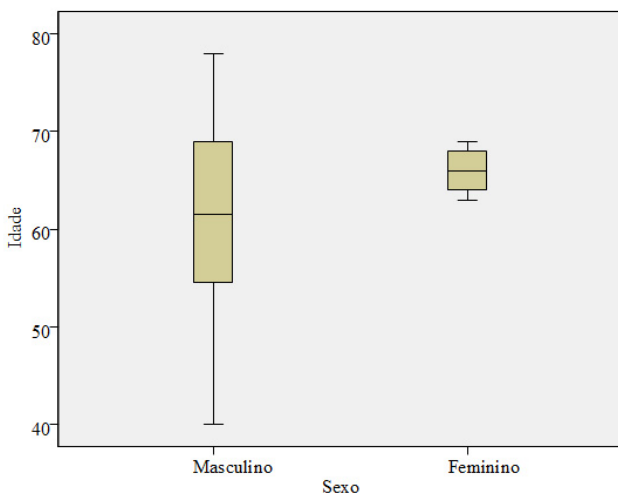


FIGURE 2 - Distribution by gender and age of patients diagnosed with adenocarcinoma of the esophagus in a period of five years (n = 37)

Most patients (67.5%) had disease in stages III and IV. Four cases (10.8%) did not have annotation of the stage.

The surgical procedures with curative intent (total gastrectomy and transhiatal esophagogastrectomy) were performed in 54% of cases. The other underwent palliative operation or did not receive surgical treatment.

The degree of tumor differentiation in five

cases was well (13.5%), 21 moderate (56.8%) and in ten undifferentiated (27%).

In immunohistochemical analysis of p16, had good agreement between pathologists regarding the evaluation of the percentage of stained nuclei (Kappa = 0.628).

Regarding the assessment of the intensity of expression of p16 in the nuclei, the agreement was fair (Kappa = 0.392).

When happened disagreement between the observers, the slides were reviewed by two pathologists until a consensus was established. For the final analysis, only the consensual results were considered.

The immunohistochemical expression of p16 was observed in only 32.4% of the cases. In six (16.2%) there was strong expression (score 3), in three (8.1%) it was moderate and in other three (8.1%) poor. In 25 cases (67.6%) loss of expression was found.

Regarding gender, there was protein expression in ten of the 32 men (31.2%) and two in five women (40%) (p = ns). In the staging, the distribution of protein expression was 50% for stage I (expression in two of four patients), 50% for stage II (expression in two of four patients), 23% in stage III (three of expression in 13 patients) and 41.7% in stage IV (expression in five of 12 patients). There was no protein expression in four patients without staging information. Statistical analysis showed no significant relationship between staging and protein expression (p = 0.485)

The distribution of p16 protein expression between the different surgical approaches were as follows: the group submitted to total gastrectomy, there was expression in three of 11 patients (27.3%); in the group undergoing palliative operation, the expression was identified in four of 11 patients (36.4%); in patients submitted to transhiatal resection, four of nine patients (44.4%) expressed the protein and the group of patients who underwent biopsy only, there was an expression of the protein in six patients (16.7%) (p = ns).

There was also no statistic significant relationship between the immunohistochemical expression of p16 and the degree of histological differentiation of tumor, when analyzed the relation to immunoreactivity score (p = 0.81).

DISCUSSION

The criteria for inclusion and exclusion of cases in this study were rigorously analyzed in order to preserve the representativeness of the sample and the compatibility of the samples with the used technique. This allowed, for example, the inclusion of material obtained from biopsies, in addition to the operative parts of resections performed. Most cases

of esophageal adenocarcinoma were diagnosed in advanced stages, with no indication for resection of the organ.

Were excluded patients undergoing neoadjuvant treatment or history or presence of other concomitant malignancy, because these situations could modify the protein expression and the status of the p16 gene^{16,20}.

Several studies show that the analysis of immunohistochemical expression of p16 protein correlates well with other methods of analysis for p16 gene alterations, such as sequencing and amplification of molecular chain reaction (PCR)^{2,3,4,9,18,19,25,31}. This scientific reason justified the use of lower cost method of analysis suitable for the objectives.

Evaluation of the expression of p16 by immunohistochemistry showed a reasonably good agreement between the observers on the criteria for positivity (nuclear staining) and according to the intensity of expression. This agreement may be due to active participation of pathologists in the various studies conducted with patients of the Hospital de Clínicas³.

Was found on literature review only one study published with clear description of the methodology used in immunohistochemical analysis of p16 in adenocarcinoma of the esophagus⁴. In this study, cases were considered negative when less than 10% of the cells showed nuclear staining. The present study used the same criteria, but added the analysis of the intensity of expression, through the modified score of immunoreactivity.

Changes in the p16 gene were studied in several premalignant lesions^{7,20,28,29} and numerous tumors^{16,23}, such as colon and rectum²⁵, pancreas¹⁰, cervix¹⁹, salivary glands¹⁸, head and neck^{9,32}, mesotheliomas¹², osteosarcomas¹⁷, gliomas¹³ bladder¹, gastrointestinal stromal tumors²⁴, ovary⁵, lung^{2,3,19}, stomach⁸ and esophagus^{4,7,13,26,27,29,31}.

Numerous changes have been described in the p16 gene, the most frequent deletions and hypermethylation^{2,3,4,7,8,9,12,13,18,19,25,29,31}. These changes are often associated with loss of immunohistochemical expression of p16 protein^{2,3,9,13,18,19,25}.

The molecular processes that accompany the progression of BE to adenocarcinoma led to several studies, establishing it as the main precursor lesion of esophageal adenocarcinoma^{4,7,28,29}. Among the causes of BE, is notable the gastroesophageal reflux disease, with rising prevalence⁶. Gurski et al.¹¹ in 2003 showed that the antireflux operation provided reversal of metaplastic and dysplastic lesions in BE to form more benign in a significant number of patients.

Changes in the p16 sequence metaplasia-dysplasia-adenocarcinoma sequence shown in some studies^{4,7,29}, correlation between the degree of

histologic and immunohistochemical expression of the prevalence of p16 protein. Our study showed no significant association ($p = 0.81$), but there seemed to be the trend of declining intensity of expression with loss of histological differentiation. It is possible that larger sample to confirm this relationship in esophageal adenocarcinoma.

Another observation that worth to be mentioned is the trend of decreasing proportion of the score of immunoreactivity in stages I, II and III (regional and local progression). In cases with stage IV (distant metastases) there was no such proportionality, suggesting that other genetic processes may play more important role in the process of metastasis, as those involved in cell adhesion and angiogenesis, for example. Further studies with larger numbers of cases and including the analysis of other genes may show the relationship between staging and genetic processes involved.

Papadimitrakopoulos et al.²⁰, in 2001, studied the expression of p16 in premalignant lesions of the upper aerodigestive tract underwent chemoprophylaxis and found no significant correlation between loss of expression with histological progression to cancer. However, no publications were found evaluating this response in BE when treated. Studies including treated cases with EB can clarify how it behaves p16 protein expression, including varying degrees of response. In adenocarcinoma of the esophagus, few studies were done but showed a high prevalence of p16 alterations in this gene (41% of genetic alterations)⁷ and significant loss of p16 protein expression (loss of expression in 86% of cases)⁴. In this study, was found loss of immunohistochemical expression of p16 in 67.6% of cases and weak expression in 8.1%. Only 24.3% of cases showed moderate to strong expression.

CONCLUSION

There was low prevalence (32.4%) of immunohistochemical expression of p16 protein in the cases analyzed, confirming the existing knowledge about the frequent changes of the p16 gene in esophageal adenocarcinoma.

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