The Gastrin-Releasing Peptide Receptor as a Marker of Dysplastic Alterations in Cervical Epithelial Cells

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Key Words

Gastrin-releasing peptide · Gastrin-releasing peptide receptor · Cervical dysplasia · Cervical cancer

Abstract

Background: Cervical cancer is a leading cancer in women worldwide. The Papanicolaou test (Pap test) remains the main screening tool; however, it produces high rates of false-negative and false-positive results. Gastrin-releasing peptide is a growth factor that has been implicated in many cancers, and its main receptor, the gastrin-releasing peptide receptor (GRPR), is nearly always expressed in cervical dysplasias and invasive carcinomas. The aim of this study was to evaluate the diagnostic potential of GRPR immunocytochemistry in detecting cervical dysplasia and invasive cancer. **Methods:** Cervical smears were collected from 66 women in Brazil and subjected to GRPR immunocytochemistry and the Pap test. GRPR and p16 immunohistochemistry were performed in biopsies if abnormalities were detected. **Results:** GRPR immunostaining sensitivity in detecting cervical lesions was 87.5% and its specificity was 76.7%. GRPR immunostaining showed 80% accuracy in identifying atypical

squamous cells of undetermined significance (ASCUS), with 88% sensitivity and 71% specificity. **Conclusion:** This is the first immunocytochemical evaluation of GRPR expression in cervical epithelial cells. This biomarker was strongly associated with cervical dysplasia and invasive cancers. GRPR immunosignaling showed high accuracy in detecting dysplasias in cells classified as ASCUS by Pap tests. Based on these results, immunocytochemistry for GRPR may be regarded as a valuable method for early detection of cervical intraepithelial neoplasia.

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Introduction

Cervical cancer is the second most common cancer in women worldwide, with about 500,000 new cases each year. The incidences are highest in developing countries, where carcinoma of the cervix is the leading cause of female cancer mortality [1]. Since the introduction of the Papanicolaou cytological screening (Pap test) there has been a significant reduction in the incidence and mortality from cervical cancer. However, many women are still

not properly screened, either because of low coverage of Pap testing or because of the limitations of this method.

The efficacy of the Pap test, a single test only 50% sensitive in detecting high-grade lesions or invasive carcinomas, is hampered by high rates of false-negative and false-positive results [2]. Technical improvements of the Pap test, such as liquid-based cytology, have not improved its sensitivity or specificity in detecting cervical intraepithelial neoplasia (CIN) compared to conventional cytology [3]. Furthermore, up to 10% of Pap tests are classified as atypical squamous cells of undetermined significance (ASCUS), i.e. specimens that cannot be clearly categorized as normal, or as displaying moderate or severe lesions, or tumors. However, experience shows that up to 10% of patients with an ASCUS classification have high-grade lesions, which are thus overlooked [4]. Therefore, it is essential to search for alternative screening and diagnostic technologies in cervical cancer. Many molecular methods have been evaluated for this purpose in recent years, among them human papillomavirus (HPV) testing, Ki-67 and p16INK4a (p16) detection [5]. The cyclin-dependent kinase inhibitor protein p16 is considered a surrogate marker of the oncogenic activities of HPV in cervical cells, and its overexpression has been well established in CIN and invasive cancer by many studies [6–9].

Gastrin-releasing peptide (GRP) is a neuroendocrine peptide shown to have growth-stimulatory effects on many types of cancer [10]. The GRP-preferring receptor (GRPR, BB2 receptor) belongs to the G-protein receptor super family and activates multiple signal transduction pathways, resulting in cell proliferation and growth [11]. GRPR is overexpressed in a wide variety of human malignancies, including prostate, breast, ovary, lung, head and neck, gastric, colon, esophageal and renal cancers, and glioma [10, 12]. In addition, we have recently described the aberrant expression of GRPR in both cervical dysplasias and invasive carcinomas. Based on the presence of this receptor in 99% of the evaluated samples, but not in non-malignant cervixes, it has been suggested that GRPR may play a role in cervical carcinogenesis [13]. These data provided the molecular basis for exploiting GRPR as a target for cervical cancer diagnosis. GRPR is already being studied for diagnostic purposes in other types of cancer, like breast and prostate cancer, with promising results [14–17]. However, to date no studies have evaluated GRPR expression in cancer using immunocytochemistry.

In this study, we aimed to investigate the sensitivity and specificity of GRPR immunocytoexpression in detecting cervical dysplasia and invasive cancer in comparison to conventional cytology. We also intended to ver-

ify whether immunocytochemical staining in cervical smears correlates to immunohistochemical signaling in the corresponding tissue specimens.

Materials and Methods

Data and Specimen Collection

Between 2009 and 2010, 66 cervical samples were randomly collected from women who attended the Gynecological Clinic at the University Hospital of Irmandade Santa Casa de Misericórdia in Porto Alegre, Brazil. The sample population consisted of 36 selected women who had been referred for colposcopy because of abnormal Pap tests (16 ASCUS, 5 CIN I, 15 CIN II–III), and 30 women undergoing routine cervical cancer screening. The study was approved by the local Research Ethics Committee (3025/09); all subjects invited to take part in the study agreed to do so, and informed consent was obtained. Patients included were aged 21–64, with a median age of 37 years. Two samples were collected from each individual by one specialist gynecologist using a spatula and cytobrush. The first smear was used for GRPR immunocytochemistry and the second for the conventional Pap test, which was performed at the hospital's pathology laboratory and interpreted by a certified pathologist. All specimens were classified according to the criteria of the World Health Organization as either normal cervix, ASCUS, mild dysplasia (CIN I), moderate dysplasia (CIN II), severe dysplasia or carcinoma in situ (CIN III), invasive squamous or adenocarcinoma. According to the Bethesda criteria, CIN I is classified as a low-grade squamous intraepithelial lesion (LSIL) and CIN II–III as a high-grade squamous intraepithelial lesion (HSIL). Cases were managed clinically according to test results. Biopsies were taken if abnormalities suggestive of cervical premalignancy or malignancy were visualized either at the time of initial specialist examination or during any subsequent colposcopy. The biopsies were evaluated by immunohistochemistry for GRPR and p16 expression.

Immunohistochemistry and Immunocytochemistry

The GRPR expression analysis was performed using a rabbit anti-GRPR polyclonal antibody (Affinity Bioreagents, Golden, Colo., USA) as primary antibody. For p16 expression, we used a p16-INK4A-specific monoclonal antibody, clone E6H4 (Dako AS, Glostrup, Denmark), primary antibody. The immunohistochemical methods have been described in our previous study [13]. Briefly, after dewaxing, inactivating endogenous peroxidase activity and blocking cross-reactions with normal serum, 4- μ m sections were incubated overnight at 4°C with a diluted solution of the primary antibody (1:50). Identification of primary antibody location was achieved by subsequent application of biotinylated antibody, streptavidin horseradish peroxidase conjugate (LSAB, Dako, and diaminobenzidine tetrahydrochloride/H₂O₂; DAB detection Kit, Dako). The procedure for the immunocytochemical analysis was identical to that described above, except that the dewaxing step in xylene and the antigen retrieval steps were omitted. A known pancreatic cancer was used as positive control and the negative control was obtained by omitting the primary antibody.

In the immunohistochemical analysis, lesions were considered positive for both GRPR and p16 if more than 10% of the cells stained moderately or strongly. Inversely, lesions were considered

Fig. 1. Immunocytochemical and immunohistochemical findings in representative cases. Immunostained cells appear as brown spots. **a** CIN III nuclear and cytoplasmic staining for GRPR expression contrasting with negative normal cells. **b** Normal epithelial cells demonstrating no GRPR expression. **c** ASCUS showing strong positivity for GRPR. **d** Diffuse cytoplasmic GRPR immunoreactivity in a CIN III biopsy. **e** Positively expressed p16 in a CIN III tissue sample. **a–c** $\times 400$. **d, e** $\times 200$.

Table 1. Evaluation of the diagnostic performance of immunocytochemical analysis (IC) of GRPR expression in detecting cervical dysplastic and neoplastic lesions

IC GRPR	Biopsy/colposcopy		Total
	lesion	control	
+	28	7	35
–	4	23	27
Total	32	30	62

Sensitivity: 87.5% (95% CI: 71.0–96.5); specificity: 76.7% (95% CI: 57.7–90.1); positive likelihood ratio: 3.75 (95% CI: 1.93–7.27); negative likelihood ratio: 0.16 (95% CI: 0.06–0.42).

negative if less than 10% of the cells stained weakly. Positive cytoplasmic or nuclear staining reactions will appear as brown spots. GRPR expression was considered positive if immunostaining was positive in at least 5 epithelial cells in every slide, based on the classification proposed by Guo et al. [18]. All samples were reviewed independently by two pathologists blinded to the previous diagnosis. In case of discrepancies, a consensus was reached with the involvement of a third pathologist.

Statistical Analysis

The sensitivity and specificity of the Pap test and GRPR overexpression in cervical smears were estimated according to the histological findings in the corresponding biopsies, considered as the

gold standard in the diagnosis of cervical disease. In the control group, however, biopsies were not performed for ethical reasons. Thus a normal colposcopy was defined as absence of neoplastic lesions. Sensitivity and specificity were calculated using PEPI version 4.0; the 95% confidence intervals (95% CIs) were calculated using a binomial distribution. Likelihood ratios were obtained using Stat Calc/epi-info, and for CIs, Taylor series were used.

Results

Immunocytochemical Staining for GRPR

Histologically confirmed biopsies consisted of 6 CIN I, 23 CIN II–III and 3 squamous carcinomas. The immunostaining analysis of the corresponding cervical smears showed GRPR positivity in the vast majority of cervical lesions. The receptor was identified in 83% of CIN I, 86% of CIN II–III and 100% of invasive carcinomas samples, mostly in dysplastic cells, but sometimes in normal epithelial cells in the same slide (fig. 1a). Most of the cells exhibited cytoplasmic staining or a combination of cytoplasmic and nuclear staining. None of the cases showed a nuclear signal only. The greater part of normal smears was negative for GRPR expression (fig. 1b), while a small number of normal smears showed immunopositivity in epithelial and inflammatory cells. The main results are summarized in table 1. GRPR immunostaining sensitivity in detecting cervical dysplas-

tic lesions and invasive cancer was 87.5% (95% CI: 71.0–96.5) and specificity was 76.7% (95% CI: 57.7–90.1). The positive likelihood ratio was 3.75 (95% CI: 1.93–7.27), while the negative likelihood ratio was 0.16 (95% CI: 0.06–0.42) (fig. 2).

Pap Test Results

The Pap test indicated altered cells in 24 smears of 31 cervical lesions. However, Pap tests matched the histological diagnosis in only 5 of 16 CIN II–III and 2 of 5 CIN I, corresponding to 32.2% of the neoplastic samples analyzed. It is important to emphasize that the Pap test failed to recognize invasive cancer cells, since 1 sample of invasive carcinoma was classified as CIN III and the other 2 were classified as ASCUS. In 32 controls with normal colposcopy, 21 of the Pap tests were normal while 11 cases were classified as abnormal (6 ASCUS, 4 CIN II–III and 1 CIN I). The results are summarized in table 2. The sensitivity of the Pap test in detecting cervical dysplastic lesions and invasive cancer was 77.4% (95% CI: 60.4–90.0) and specificity was 65.6% (95% CI: 48.1–80.4). The positive likelihood ratio was 2.25 (95% CI: 1.35–3.77), while the negative likelihood ratio was 0.34 (0.17–0.69) (fig. 2).

ASCUS Lesions

Among 63 patients analyzed, the Pap test classified 16 lesions as ASCUS. Of those, 7 related to normal colposcopies in the control group. The other 9 proved to be 1 CIN I, 6 CIN II–III and 1 invasive squamous carcinoma by histological analysis. GRPR immunostaining was performed in 15 of those patients classified as ASCUS. The marker was positive in 7 of 8 abnormal samples (fig. 1c) and negative in 2 of 5 nonmalignant lesions, which led to a test accuracy of 80%. The sensitivity was 88% and the specificity 71%, while the positive and negative predictive values were 77 and 83%, respectively. Of note, 2 lesions classified as ASCUS turned out to be invasive carcinomas, and GRPR showed strong immunostaining in those samples.

Immunohistochemical Staining for GRPR and p16

In 25 patients with confirmed dysplasias or invasive cervical carcinomas, immunohistochemical staining for GRPR was performed. In 88% of the samples, the expression of GRPR was positive, generally with a strong signal in dysplastic and neoplastic epithelia, clearly distinct from adjacent normal epithelia or stroma cells (fig. 1d). Most of the samples exhibited a predominantly cytoplasmic staining pattern.

Fig. 2. Graphic representation of proportions and error bars representative of the sensitivity (Se) and specificity (Sp) of the immunocytochemical analysis of GRPR expression (black circle) and the Pap test (white square) in detecting cervical lesions, and positive and negative likelihood ratios (LR+, LR–), with the respective 95% CIs.

Table 2. Evaluation of the diagnostic performance of the Pap test in detecting cervical dysplastic and neoplastic lesions

Pap test	Biopsy/colposcopy		Total
	lesion	control	
+	24	11	35
–	7	21	28
Total	31	32	63

Sensitivity: 77.4% (95% CI: 60.4–90.0); specificity: 65.6% (95% CI: 48.1–80.4); positive likelihood ratio: 2.25 (95% CI: 1.35–3.77); negative likelihood ratio: 0.34 (95% CI: 0.17–0.69).

p16 immunohistochemical staining was performed in 26 neoplastic tissues. Intense positive staining was found in 88.4% of the samples. Most dysplastic and neoplastic cells showed cytoplasmic staining (fig. 1e). In both GRPR and p16, the markers were expressed in CIN I, CIN II–III and invasive cancer. We did not observe higher signaling in more advanced lesions. The degree of agreement between GRPR immunostaining in cervical smears and the corresponding cervical lesions was 88%. Only in 3 CIN II–III did we find no GRPR expression, whereas immunocytochemical analysis yielded positive tests.

Discussion

The Pap test is currently the method of choice for detecting cervical cancer. The Pap test is a subjective method that has remained substantially unchanged for many decades. There are several concerns, however, regarding its performance. The reported sensitivity of a single Pap test is low and shows wide variation (30–87%), and the specificity of a single Pap test might be as low as 86% in a screening population [2]. In recent years, technological advances in sample collection and processing have been achieved in cervical screening programs, with a positive impact on the detection of cervical lesions. However, the morphological interpretation of cytological tests still has limitations. It has been demonstrated that misinterpretation and interobserver discrepancies are common, specifically within the ASCUS and LSIL cytology categories [19]. A significant percentage of Pap smears characterized as ASCUS or LSIL are actually high-grade lesions [3]. Thus, a reliable method for diagnosing cervical disease that is independent of, or works in conjunction with, the conventional Pap test is needed. A wide array of potential biomarkers has been tested to test their diagnostic usefulness in the evaluation of cervical cancer and its precursors.

The GRPR is emerging as a very promising target for both cancer diagnosis and treatment. Over the last decades, several lines of experimental evidence have suggested that GRP may play a role in cancer development [20–22]. GRP has been recognized as an autocrine mitogen based on the detection of the growth factor and its cognate receptor in the same tissue, resulting in proliferation [23]. Recent data show that GRP also has paracrine and endocrine effects and functions as a morphogen and a proangiogenic agent [24]. Additionally, inhibition of GRPR was demonstrated to interfere with other relevant growth factor pathways such as the epidermal growth factor- and vascular endothelial growth factor-dependent signaling pathways [25, 26]. We recently reviewed the GRPR expression status in human cancers. These receptors have been identified in many malignancies, including prostate, breast, ovary, lung, head and neck, gastric, colon, esophageal and renal cancers, but no data have been reported for cervical carcinomas [10]. In an earlier immunohistochemical study, we found aberrant GRPR expression in dysplastic squamous lesions and invasive squamous carcinomas of the cervix, in contrast to low expression in normal epithelia and endocervices. Based on those findings, we postulated that GRPR could be involved in cervical carcinogenesis [13]. These results

prompted us to continue investigating GRPR immunosignaling in cervical smears.

The first objective of this study was to evaluate the feasibility of GRPR immunocytochemistry in cervical smears since this technique had not been previously described for any tumors. We believe immunocytochemistry is a highly interesting approach since it is a simple and affordable method that can be performed in a nonsophisticated environment. Additionally, it offers the advantage of enabling a patient to avoid a surgical intervention. We were able to perform GRPR immunocytochemical staining successfully in cervical smears using the same fixative method as that required for the Pap test. Of the 66 samples analyzed in this study, only 3 could not be interpreted due to failures in the staining technique.

In this study, the staining intensity was not graded to avoid subjective findings. We only counted the number of immunopositive cells in a medium-power microscopic field as described by Guo et al. [18] for p16 expression. Since this is the first description of GRPR immunocytochemistry in cervical smears, we had no previous parameters with GRPR to compare with. Therefore, we based our research on extensive data from p16 immunocytochemical studies. There is no consensus, so far, on a standard methodology, especially regarding the interpretation of cervical smear results. For the time being there are no clear-cut arguments for establishing threshold values above which a sample becomes 'positive'. In the systematic review and meta-analysis by Klaes et al. [27], cut-off for p16 positivity ranged from 1 cell to more than 30% of cells.

GRPR immunocytochemical staining was found in the great majority of cervical lesions; 83% of CIN I, 86% of CIN II–III and 100% of invasive carcinomas samples. Compared to p16, the most studied biomarker of cervical dysplasia, this expression was higher in CIN I and equivalent in CIN II–III, according to data reported in a meta-analysis of p16 expression. Although the positivity cut-off varied, the average p16 positivity for CIN I and CIN II–III was 45% (37–57%) and 89% (84–95%), respectively [25]. Most GRPR-positive cells predominantly exhibited cytoplasmic staining. This finding is similar to our previous results with GRPR immunohistochemistry. However, the features of nuclear versus cytoplasmic staining have not yet been analyzed systematically, and are currently not considered to be relevant by the majority of authors [27]. Interestingly, in abnormal smears, GRPR immunosignaling was not limited to dysplastic cells, but could also be seen in some normal epithelial cells. On the other hand, GRPR expression was seldom detected in normal smears.

This confirms our previous findings, i.e. normal cells adjacent to lesions exhibited GRPR immunosignaling, contrasting to no signaling in nonmalignant tissues. We concluded that the presence of GRPRs in those cases could represent an early molecular event in cervical carcinogenesis.

In patients with confirmed dysplasia or invasive cervical carcinomas, immunohistochemical staining for GRPR showed 88% positivity, generally with a strong signal in dysplastic and neoplastic epithelia, clearly distinct from adjacent normal epithelia or stroma. This result was superior to that of our previous study, in which 78.4% of the tissues exhibited such moderate or strong diffuse GRPR staining. p16 immunohistochemistry was also performed in the present study and yielded virtually the same results as GRPR expression, i.e. 88.4% positive results.

GRPR was homogeneously expressed through the different grades of CIN and squamous carcinomas in both smears and tissue specimens. We did not find a larger number of positive cells to be associated with increased severity of the lesions. This reproduced our earlier results, as we found no significant differences in GRPR expression among the dysplastic and neoplastic specimens. Data in the literature are controversial: some authors were able to associate a higher incidence of GRPRs with poorly differentiated or more aggressive lesions [28–30], while others did not find GRPR expression to be associated with disease progression [31, 32].

In our previous work on cervical tissues, we demonstrated that GRPR is aberrantly expressed in cervical squamous dysplasia and neoplasia, but is absent in nonmalignant ectocervices [13]. The use of biopsies has the advantage of preserving tissue architecture and cellular morphology, enabling immunoreactions to be attributed to specific subpopulations; however, they require invasive procedures. Thus, another important goal of this study was to verify whether immunocytochemical staining in cervical smears correlated to immunohistochemical signaling in the corresponding tissue specimens, so that the GRPR status could be evaluated in a noninvasive manner. The degree of agreement between GRPR immunostaining in cervical smears and the corresponding cervical lesions was 88%. GRPR expression was absent in only 3 CIN II–III samples, contrasting with a positive immunocytochemical test.

Despite several limitations, the Pap test remains the most widely used method for cervical cancer screening. In the current study, it was compared with the histologic diagnosis in biopsy samples taken from the same pa-

tients. The Pap test indicated altered cells in 24 smears of 31 cervical lesions. Its sensitivity was 77.4% and its specificity was 65.6%. A systematic review of the sensitivity and specificity of conventional cervical cytological tests found biases in many studies and wide variation in their results. Evaluating the studies with the best methodology and valid controls, the sensitivity of a conventional Pap test for the diagnosis of CIN I or worse ranged from 30 to 87% with 86–100% specificity [33]. According to the current literature, there is clearly some correlation between abnormal cytological results and subsequent histological findings in biopsies, but a direct correspondence is found in only about half of the patients [34]. Consistent with this information, our Pap test results matched the histological diagnosis in only 32.2% of the neoplastic samples analyzed. Of note, 3 invasive squamous carcinomas were underdiagnosed by the Pap test.

A major objective of this study was to compare the diagnostic utility of GRPR immunocytochemistry with that of Pap test. Figure 2 shows the sensitivity, specificity, and the positive and negative likelihood ratios of both tests. There is a clear diagnostic advantage for GRPR expression in all measures. The ability of GRPR immunostaining to detect cervical lesions was 10.1% higher than that of the Pap test, with a gain of 11.1% in specificity. The likelihood ratios are used for assessing the value of performing a diagnostic test. They use the sensitivity and specificity of the test to determine whether a test result usefully changes the probability that a disease exists. As expected, the positive likelihood ratio of GRPR immunocytochemistry was superior to that of the Pap test while the negative likelihood ratio was inferior. GRPR immunostaining showed higher diagnostic utility compared with the Pap test, although the sample size was too small to produce statistically significant results. We believe that further studies including a larger number of patients are needed.

Finally, another important issue was to evaluate lesions classified as ASCUS with respect to GRPR expression. We had a biased higher proportion of ASCUS compared to the normal population since we are a reference colposcopy unit. Seven of 16 samples classified as ASCUS had been diagnosed as normal colposcopies in the control group, and 9 had been identified as dysplastic or invasive lesions proven by histological analysis. It is well recognized that the Pap test is less sensitive when changes consistent with ASCUS are seen [35]. Additionally, the diagnosis of ASCUS has considerable interobserver variability, even among expert pathologists. In the ASCUS/LSIL study, the diagnostic agreement was of only 55% of the

submitted ASCUS cases [34]. Unfortunately, the low sensitivity of the Pap test in this regard affects millions of women because up to 5% of all Pap tests will be classified as ASCUS, and only a small subset of those patients (5–17%) will have a biopsy proving CIN II–III lesions, which definitely require treatment [36]. For this reason, methods for identifying those patients erroneously classified as ASCUS following Pap tests but more likely to have dysplasia are needed. GRPR immunocytochemistry showed high accuracy (80%) in the ASCUS diagnosis, either in detecting the presence or absence of lesions. The sensitivity was 88% and the specificity 71%, while the positive and negative predictive values were 77% and 83%, respectively. In the present work, 2 patients referred for colposcopy because of an ASCUS diagnosis had invasive carcinomas. GRPR showed strong immunostaining in those samples.

This study had limitations, mainly related to sample size, although the major objective was to assess whether GRPR expression could be used as a potential diagnostic tool. We believe that a cohort study that includes at least 1,000 patients is necessary to demonstrate a significant role of GRPR expression as a marker of dysplastic cervical lesions.

Conclusion

To the best of our knowledge, this is the first immunochemical study evaluating GRPR expression in cervical epithelial cells. The biomarker was strongly associated with cervical dysplasia and invasive cancers. GRPR immunosignaling showed high accuracy in detecting precancerous lesions among Pap tests classified as ASCUS. Based on these results, immunocytochemistry for GRPR expression may be regarded as a valuable test for the early detection of CIN.

Acknowledgements

This research was supported by the National Council for Scientific and Technological Development (CNPq; grant No. 303703/2009-1 to R.R.), the National Institute for Translational Medicine (INCT-TM) and the South American Office for Anti-cancer Drug Development.

Disclosure Statement

The authors have no conflicts of interest to declare.

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