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Clinical importance of p16, Ck7 expression and histological findings in uterine cervix immature metaplasia

Importância clínica da expressão de p16, Ck7 e achados histológicos na metaplasia imatura do colo uterino

Importancia clínica de la expresión de p16 y Ck7 y hallazgos histológicos en la metaplasia inmadura del cuello uterino

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ABSTRACT

Objective: To investigate potential molecular markers as prognostic factors in immature squamous metaplasia (ISM) in the cervix and to evaluate the clinical significance of finding typical and atypical ISM in the cervical epithelium of the patient. **Methods:** A total of 79 cases were selected in a retrospective cross-sectional study (2011-2019). Women with typical or atypical ISM were evaluated according to sociodemographic, cytological, and colposcopy criteria. The biopsy samples were evaluated by anatomopathological and immunohistochemical analysis, in which the expression of P16INK4a (p16) and Ck7 was investigated. **Results:** Cytological analysis showed a higher frequency of atypical squamous cells of undetermined significance with high-grade lesions. Regarding colposcopy analysis, both groups presented similar results. The negativity of p16 and Ck7 expression was associated with a favorable outcome. However, no correlations were found between the expression of p16 and Ck7 and typical or atypical ISM. In addition, a kappa value of 0.087 was obtained, confirming the significant interobserver variability of the study. **Conclusion:** The absence of p16 and Ck7 can be used as biomarkers of favorable prognosis, but they did not show correlations with classifications of typical or atypical ISM in the cervix. Therefore, other effective methodologies for screening this patient profile should be further investigated.

Keywords: Immature Squamous Metaplasia (ISM), Uterine cervix, p16, Ck7, Biomarkers.

RESUMO

Objetivo: Investigar potenciais marcadores moleculares como fatores prognósticos na metaplasia escamosa imatura (ISM) no colo uterino e avaliar o significado clínico de encontrar ISM típico e atípico no epitélio cervical da paciente. **Métodos:** Foram selecionados 79 casos em estudo transversal retrospectivo (2011-2019). As mulheres com ISM típico ou atípico foram avaliados quanto a critérios sociodemográficos, citológicos e colposcopia. As amostras de biópsia foram avaliadas por análise anatomopatológica e imunohistoquímica, nas quais foi investigada a expressão de P16INK4a (p16) e Ck7. **Resultados:** A análise citológica mostrou maior frequência de células escamosas atípicas de significado indeterminado com lesões de alto grau. Quanto à análise da colposcopia, ambos os grupos apresentaram resultados semelhantes. A negatividade da expressão de p16 e Ck7 foi associada a um desfecho favorável. Porém, não foram encontradas correlações entre a expressão de p16 e Ck7 e o ISM típico ou atípico. Além disso, foi obtido um valor kappa de 0,087, confirmando a significativa variabilidade interobservador do estudo. **Conclusão:** A ausência de p16 e Ck7 pode ser utilizada como biomarcadores de prognóstico favorável, mas não mostraram correlações com

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classificações de ISM típico ou atípico no colo uterino. Logo, deve-se continuar investigando outras metodologias eficazes para triagem desse perfil de pacientes.

Palavras-chave: Metaplasia Escamosa Imatura (ISM), Colo uterino, p16, Ck7, Biomarcadores.

RESUMEN

Objetivo: Investigar potenciales marcadores moleculares como factores pronósticos en la metaplasia escamosa inmadura (ISM) en el cuello uterino y evaluar la importancia clínica del hallazgo de ISM típica y atípica en el epitelio cervical de la paciente. **Métodos:** Se seleccionaron un total de 79 casos en un estudio transversal retrospectivo (2011-2019). Las mujeres con ISM típica o atípica fueron evaluadas según criterios sociodemográficos, citológicos y de colposcopia. Las muestras de biopsia se evaluaron mediante análisis anatomopatológico e inmunohistoquímico, en el que se investigó la expresión de P16INK4a (p16) y Ck7. **Resultados:** El análisis citológico mostró una mayor frecuencia de células escamosas atípicas de significado indeterminado con lesiones de alto grado. En cuanto al análisis colposcópico, ambos grupos presentaron resultados similares. La negatividad de la expresión de p16 y Ck7 se asoció con un resultado favorable. Sin embargo, no se encontraron correlaciones entre la expresión de p16 y Ck7 y el ISM típico o atípico. Además, se obtuvo un valor de kappa de 0,087, lo que confirma la significativa variabilidad interobservador del estudio. **Conclusión:** La ausencia de p16 y Ck7 pueden ser utilizados como biomarcadores de pronóstico favorable, pero no mostraron correlaciones con las clasificaciones de ISM típico o atípico en el cuello uterino. Por lo tanto, se deben investigar más a fondo otras metodologías efectivas para el cribado de este perfil de paciente.

Palabras clave: Metaplasia Escamosa Inmadura (MSI), Cuello uterino, p16, Ck7, Biomarcadores.

INTRODUCTION

Cervical cancer is one of the principal causes of morbidity and mortality among women worldwide, being classified as the second leading common malignancy (LIKHITA K, et al., 2024). Although the overall incidence has declined since the 1970s, it was observed that women between the ages of 30 and 44 showed an increase in the rate of 1.7% per year between 2012 and 2019 (SIEGEL et al., 2024).

The PAP smear, while the gold standard for cervical cancer screening, has its limitations, including low sensitivity and a high rate of false negative results. However, the field is evolving, studies using precursor screening have shown that cytology analysis can reduce the incidence of invasive cervical cancer (KRISHNAMURTHY A e RAMSHANKAR V, 2020; LIKHITA K, et al., 2024). Introducing new technologies, such as Human Papillomavirus (HPV) DNA tests and biomarkers, is promising since these tools enhance decision-making in screening in the future of cervical cancer detection (LIKHITA K, et al., 2024; SEKAR PKC, et al., 2024).

Biomarkers are mainly used for molecular screening tests and are clinically associated with HPV infections. In this way, P16INK4a and CK7 are valuable biomarkers for the study of cervical cancer. CK7, a squamous-columnar junction (SCJ) cell marker, is often overexpressed in this region, which is susceptible to HPV infection (YAN Q, et al., 2024). Additionally, p16INK4a overexpression is commonly observed in cervical intraepithelial neoplasia and cancer, particularly in HPV-associated tumors (AGOFF et al., 2003). The main benefit of this technique is better risk assessment and screening for HPV infection (SEKAR PKC, et al., 2024). Mainly because most precursor lesions regress spontaneously, avoiding unnecessary interventions and more effective follow-up (ACCP, 2004; SEKAR PKC, et al., 2024).

One of the main challenges within cervical cancer detection is the diagnostic reproducibility of the histopathological examination, since it shares histological features with cervical intraepithelial neoplasia (CIN) (KOLIOPOULOS G, et al., 2017). In addition, diagnosis precursor methods assist in identifying reactive mimics, such as atypical squamous metaplasia (ASM), immature squamous metaplasia (ISM), reactive/reparative atypia (RA), and atrophy, among others (IRANPOUR M, et al. 2021).

Squamous metaplasia is a normal process that happens at different rates in the different areas of the cervix, with cells found at different degrees of maturity (GIROUX V e RUSTGI AK, 2017). The mature squamous epithelium presents no risk of complications (PRENDIVILLE W e SANKARANARAYANAN R, 2017). However, the development of the immature metaplastic epithelium can result in either a mature metaplastic squamous

epithelium or an atypical dysplastic epithelium (GIROUX V e RUSTGI AK, 2017). Atypical immature metaplasia represents a group of lesions with heterogeneous oncogenic potential (IMAI Y, et al., 2022), that may regress to normal, persist as dysplasia, or progress to invasive neoplasia after years (SELLORS JW e SANKARANARAYANAN R, 2003).

To better understand typical and atypical immature squamous metaplasia, cytological, colposcopic, and prognostic aspects were correlated with two biomarkers: P16INK4a and CK7; to obtain determining indicators for clinical diagnosis. This work aimed to evaluate the clinical importance of the finding of typical and atypical immature squamous metaplasia in the cervical epithelium of patients being monitored at the Center for Prevention of Gynecological Diseases at UNIFESP and the immunohistochemical expression of P16INK4a proteins (p16) and CK7 in these epithelia.

In this sense, the objective of this work is to evaluate the clinical importance of the finding of typical and atypical immature squamous metaplasia in the cervical epithelium of patients being monitored at the Center for Prevention of Gynecological Diseases at UNIFESP and the immunohistochemical expression of P16INK4a proteins (p16) and CK7 in these epithelia.

METHODS

Study Design

The study is a retrospective cross-sectional study using data and samples of uterine cervical epithelium biopsies of patients with confirmed diagnosis of ISM. The samples were collected between the years 2011 and 2019, at the Lower Genital Tract Pathology Outpatient Clinic of the Department of Gynecology of the Federal University of São Paulo, in Brazil. Research Ethics Committee of the Federal University of São Paulo - Escola Paulista de Medicina (UNIFESP/EPM) approved this study under protocol number 1367/2019, CAAE nº 27280819.9.0000.5505, and all patients were kept anonymous. Human samples were analyzed following international and national regulations following the Declaration of Helsinki.

Patients' selection

The study had a consecutive series of 79 selected cases. The inclusion criteria were uterine cervix samples from women over 25 with a histopathological diagnosis of typical or atypical immature squamous metaplasia, with or without associated cervical intraepithelial neoplasia. The exclusion criteria were pregnant or lactating women; diagnosis of mature squamous metaplasia; age less than 25 years at the time of diagnosis; lack of patients' paraffin block; patients with no clinical data in the medical record; clinical follow-up less than six months. The variables included in the study were patients' age, smoking, immunological status, cytological result before diagnosis, and colposcopic findings.

Anatomopathological analysis

This analysis occurred after the preparation of the slides from the paraffin-embedded tissue blocks, cut at 4mm thickness, obtained from cervical fragments from the directed biopsies in the abnormal transformation zone or surgical procedures. Based on the slides, the initial diagnosis was reviewed by Richart's classification, dividing the cases into negative, typical immature metaplasia, atypical immature metaplasia, and possible associated diagnoses (CIN 1, CIN 2 and CIN 3), by a pathologist according to previously established classical criteria (HERRINGTON CS, et al., 2020). A histopathological diagnosis of CIN 3, adenocarcinoma in situ, or invasive carcinoma after a minimum clinical follow-up of 6 months counted from the assessed sample was considered an unfavorable clinical outcome (positive). The patients' previous colpocytology slides analysis followed the Bethesda Classification.

Immunohistochemical assay

The immunohistochemistry technique identified the immunoexpression of p16 and CK7, using primary antibodies Anti-p16INK4a (Clone: G175-405) and mouse monoclonal antibody Anti-human Cytokeratin 7 (Clone OV- TL 12/30) and a secondary biotinylated antibody from the REVEAL kit - Biotin-Free Polyvalent

DAB from Spring Bioscience. The analyses were conducted in the Immunopathology Laboratory and Special Techniques of the Federal University of São Paulo Pathology Department. The immunohistochemistry technique used the primary antibody incubation protocol where samples were incubated with the antibody "overnight" for 18 hours at a controlled temperature of 2 to 8°C.

Then, the secondary antibody was applied to the tested material for 20 minutes at room temperature. After, there was an incubation time with the streptavidin-peroxidase complex from the REVEAL - Biotin-Free Polyvalent DAB kit from Spring Bioscience in a humid chamber at room temperature (18 to 22 °C) for 20 minutes. The technique was revealed by adding complex 2, 3, diaminobenzidine + hydrogen peroxide substrate within 10 minutes until the color turned brown and then counterstained with Harris hematoxylin. For the interpretation of p16 immunoexpression, we used the 2012 LAST consensus recommendations (12): Negative (no or focal immunoexpression) and Positive (diffuse nuclear positivity and cytoplasm of the complete at least basal third of the epithelium). For Ck7, the following consensus were applied:

1. Pure negative is negativity in 100% of the cells.
2. Negative in most cells, i.e., more than 50% of cells, with weak focal positivity in coilocytes.
3. Negative in most cells with focal positivity and moderate to strong positivity in coilocytes.
4. Positive in most cells, i.e., more than 50% of cells, with a homogeneous pattern and weak intensity.
5. Positive in most cells, in a homogeneous pattern, with moderate to strong intensity.
6. Positive in most cells, heterogeneous pattern, with weak intensity.
7. Positive in most cells, heterogeneous pattern, moderate to strong intensity.

For the comparison between the clinical outcomes of the patients, expression of P16 and CK7 and the opinion of the pathologists, the analysis categories were grouped as: Negative (1, 2, 3), Positive (4, 5, 6, 7) and no data.

Statistical Analysis

Qualitative variables were compiled through a clinical profile of the patients, with a descriptive analysis by constructing absolute and relative frequency tables. The pathologists' opinion, the association between the result of markers for p16 and CK7 by immunohistochemistry, and the clinical outcome of the patients were compared from the inferential analysis using Pearson's Chi-square test or Fisher's Exact test extension. Values of $p < 0.05$ indicated statistical significance. The data were analyzed in the Jamovi statistical software version 2.3.18.

RESULTS

The study population had a homogeneous distribution between typical and atypical squamous metaplasia groups. Most of the women analyzed were between 25 and 45 years old, with parity between 0 and 4, non-smokers, and without immunosuppression (**Table 1**). Smoking habits and immunosuppression were also evaluated, but no associations were found to typical or atypical classifications of ISM (**Table 1**).

Table 1 - Association between patients' sociodemographic data and the typical and atypical ISM.

Variable	Typical	Atypical	P-value*
Age			
25 ⊢ 45	21	27	0.536
45 ⊢ 65	15	11	
≥ 65	3	2	
Total	39	40	
Parity			
0 ⊢ 4	30	32	0.092
4 ⊢ 8	1	5	
≥ 8	1	1	
No information	7	2	
Total	39	40	
Previous Smoking			
Yes	3	5	0.803
No	23	21	
No information	13	14	
Total	39	40	
Immunosuppression			
Yes	10	7	0.422
No	29	33	
Total	39	40	

Source: Carvalho MCC, et al., 2025.

Interobserver variability was confirmed in our study through kappa with a value of 0.087. It demonstrates the need for inter-pathologist consensus, indicating that the accurate prognosis of our patient with this diagnosis is uncertain, which makes decision-making difficult. Cytology analyses showed no significant differences in atypia degree when correlating typical or atypical ISM to cytopathological diagnosis. As shown in table 2, most cases evaluated by the study were diagnosed as atypical squamous cells of undetermined significance with high-grade lesions (ASC-H) (atypical squamous cells of undetermined significance that cannot exclude high grade intraepithelial injury).

Only one case of the study was diagnosed as adenocarcinoma in situ, 45 cases were cytologically diagnosed as non-neoplastic or possibly non-neoplastic lesions (ASC-US, ASC-H, AGC, AGC-H, AOI and AOI-H) and 21 cases were cytologically diagnosed as low- or high-grade lesions (LSIL and HSIL). Among 17 cases classified as HSIL by cytology, 12 were atypical ISM (**Table 2**). As for colposcopy analyses, 33 cases showed normal or minor findings and no significant differences in atypia degree were found when correlating typical or atypical ISM to colposcopy evaluation (**Table 2**). Unfavorable prognosis was defined as the outcome of invasive carcinoma or adenocarcinoma after at least 6 months of follow-up.

Table 2 - Cytology and colposcopy results for typical and atypical ISM.

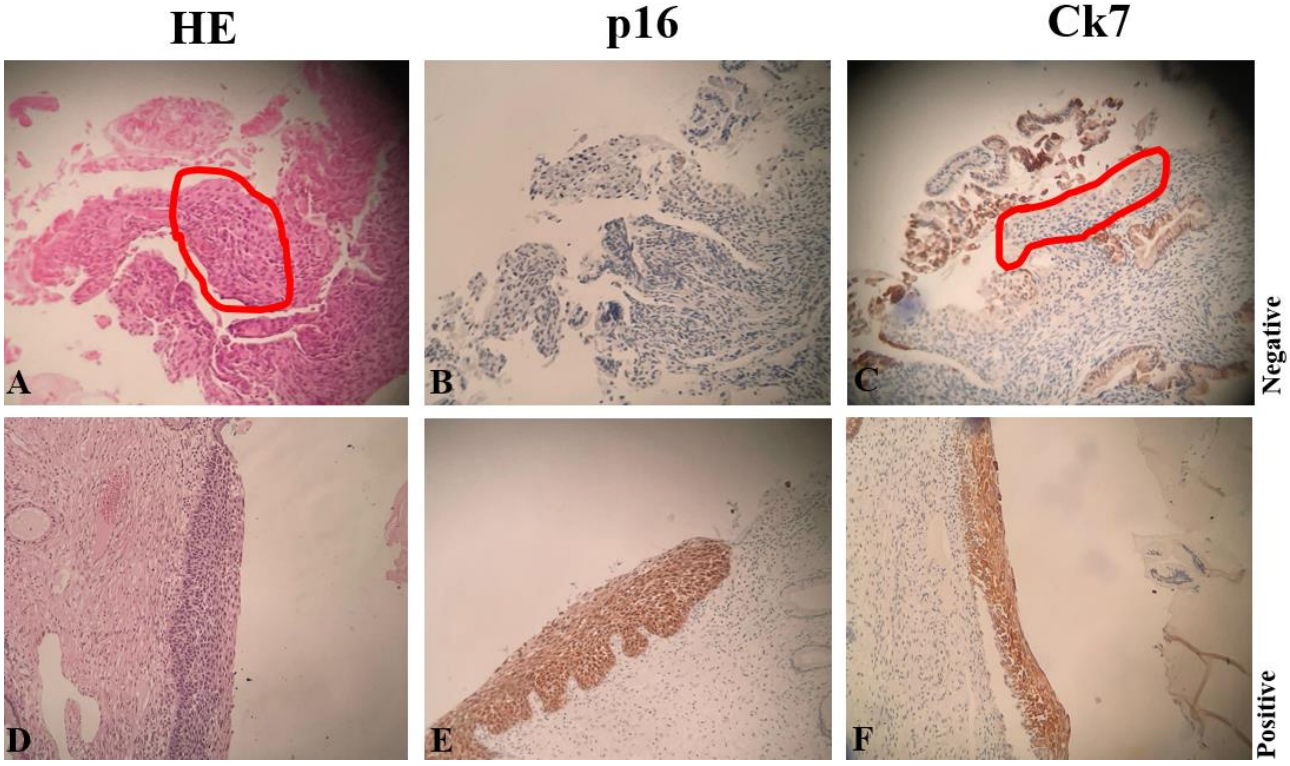
Variable	Typical	Atypical	P-value
Cytology			
Negative	8	4	0.610
ASCUS	3	3	
LSIL	2	2	
AGC	3	1	
AOI	1	1	
ASCH	15	15	
HSIL	5	12	
AGC-H	1	0	
AOI-H	1	1	
Adenocarcinoma in situ	0	1	
Total	39	40	

Variable	Typical	Atypical	P-value
Colposcopy			0.785
Normal	2	0	
Minor findings	15	16	
Major findings	9	13	
Non-specific findings	2	2	
Miscellaneous	9	7	
No information	2	2	
Total	39	40	

Note: ASCUS - Atypical Squamous Cells Of Undetermined Significance, Possibly Non-Neoplastic; LSIL - Low-Grade Intraepithelial Lesion; AGC - Atypia Of Glandular Cells; AOI - Atypia Of Undefined Origin; ASCH - Atypical Squamous Cells Of Undetermined Significance, Which Cannot Be Excluded From A High-Grade Lesion HSIL - High-Grade Intraepithelial Lesion; AGC-H - Atypia Of Glandular Cells, Which Cannot Be Excluded From High-Grade Lesions; AOI-H - Atypia Of Undefined Origin That Cannot Exclude A High-Grade Lesion. **Source:** Carvalho MCC, et al., 2025.

As shown in immunohistochemistry analyses (**Figure 1**), positive and negative staining of p16 and Ck7 were correlated atypia degree and clinical prognosis. Expression of p16 and CK7 had no significant correlations found to atypia degree in ISM (**Table 3**). However, among all samples analyzed, most had a favorable prognosis for both atypia degree groups, in which 51 were negative to Ck7, 50 negative to p16 and 43 negative to both biomarkers (**Table 3**). Thus, when correlating the positivity of markers with the unfavorable outcome, no statistical significance was found.

Figure 1 - Cases selected for analysis of uterine cervix histology showing immature squamous metaplasia. In (a) atypical immature metaplasia with negative p16 (b) and ck7 (c) (highlighted with the red circle). In (d) atypical immature metaplasia with positive p16 (e) and Ck7 (f) throughout the thickness of the epithelium. (x100 magnification).



Source: Carvalho MCC, et al., 2025.

Table 3 - Immunohistochemistry results for typical and atypical ISM.

Variable	Typical			Atypical		
	Favorable	**Unfavorable**	**p-value**	**Favorable**	**Unfavorable**	**p-value**
CK7			1,000			
Negative	20	2	-	31	3	0.154
Positive	5	0		4	2	
Total	25	2		35	5	
P16			0.279			
Negative	22	1	-	28	5	0.565
Positive	3	1		7	0	
Total	25	2		35	5	
CK7/P16			1,000			
Both Negative	19	1	-	24	3	NA
Both Positive	2	0		0	0	
Total	21	1		24	3	

Source: Carvalho MCC, et al., 2025.

DISCUSSION

Different studies look for minimizing contradictory results in the search for a method that directly or indirectly identifies women with a higher risk of developing high-grade lesions from the diagnosis of immature squamous metaplasia. This study evaluated clinical and immunohistochemical markers differences in typical and atypical immature metaplasia to improve patients' treatment and follow-up without causing unnecessary interventions. One of the challenges observed here was the large interobserver variability, confirmed in our study through the kappa with a value of 0.087. A similar analysis compared 1790 biopsy specimens of different lesions among 12 pathologists showed that atypical metaplasia fell into the category of the difficult call. The comparison among the groups showed a 0.658 generalized Kappa (MALPICA A, et al., 2005).

The difficulty encountered by both the pathologist and the gynecologist in diagnosing immature squamous metaplasia is due to the lack of identification of unique features that assist in making decisions for the best management of patients, whether this is more conservative or invasive. IMAI Y, et al. (2022) suggested reclassifying atypical immature metaplasia based on combining nuclear features, using hematoxylin and eosin staining without immunohistochemical markers. Following this analysis, they suggested a diagnosis based on nuclear features, which would increase accuracy and be a viable option when immunohistochemistry is unavailable. However, they highlighted that there would still be a considerable portion of underdiagnoses that would require the use of biomarkers such as p16 and Ki-67 for accurate reclassification (IMAI Y, et al., 2022)

When analyzing the degree of cytological atypia in typical and atypical metaplasia in both groups, the cytology result was higher for the group classifying the cells as ASC-H analyzed cases with ASC-H reports after a pap smear (MOKHTAR GA, et al., 2008). The samples classified as immature squamous metaplasia showed a delicate chromatin pattern along with dense cytoplasmic differentiation, and through analysis and their morphology demonstrated similarity to other studies showing that immature squamous metaplastic cells are similar to atypical squamous cells (MOKHTAR GA, et al., 2008).

Among the approaches used, the colposcopy results mostly found minor findings, and the typical and atypical metaplasia groups had similar results with no statistical significance. However, it is important to consider that colposcopy as a diagnostic test can also present false positive and false negative results, especially when no cytology results are available (BASU P, et al., 2013).

Through the LAST consensus, the use of p16 for diagnostic purposes has been well established since immature metaplasia with a positive p16 stain is equivalent to a high-grade lesion (DARRAGH TM, et al., 2012). Moreover, it is used in routine medical practice to double-detect protein expressions, such as p16 and ki-67 (HWANG H, et al., 2020). Therefore, the double staining method using p16 and ki-67 may be interesting for patients with cytological diagnosis of ASC-US, and ASC-H, as pointed out by Wu Y, et al. (2017) after a study using 259 cases. However, despite this implementation, the correlation of this biomarker for the

prognostic purpose is still a challenge, so adding squamocolumnar junction biomarkers can be an essential tool to achieve this purpose. Van Der Marel J, et al. (2014) compared p16, CK17, and CK7, in immature metaplastic cells, classifying atypical immature metaplasia as a category that causes diagnostic problems.

When analyzing the results from the biomarkers, atypical squamous metaplasia was distinguished from typical by nuclear enlargement and positivity for p16, Ck17, and sometimes Ck7 (VAN DER MAREL J, et al., 2014). However, the results obtained in this study showed no pattern difference between the biomarkers analyzed.In comparing the patterns of atypical squamous metaplasia and high-grade intraepithelial lesions, another study demonstrated that atypical squamous metaplasia shows positive immunoexpression for Ck17 and p63 and for p16 negative. This study also suggested that labeling Ck17 and p16 biomarkers could separate these classifications (REGAUER S e REICH O, et al., 2007).

In the analysis performed of the expression of p16 and CK7, it was impossible to observe a relationship significantly related to the outcome of typical or atypical immature squamous metaplasia. However, it was possible to associate the favorable outcome with the negativity of the markers. Goyal A, et al. (2020) used p16 to characterize premalignant cervical lesions. They found that it rarely shows strong and diffuse positivity in squamous metaplasias (GOYAL A, et al., 2020), which corroborates the results found in this study, where only 16.4% of patients with immature squamous metaplasia were positive for this marker. At the same time, Iranpour et al. (2021) analyzed pathology reports of 50 cases classified as atypical squamous metaplasia and 50 cases with CIN and presented that p16 immunolabeling in atypical squamous metaplasia samples was 100% negative, and 50.8% of cases classified as CIN showed this positivity (IRANPOUR M, et al., 2021). In our study, 17.5% of patients with atypical immature metaplasia were positive for p16.

The association between p16 and cervical lesions varies from one study to another, depending on the degree of lesions analyzed. Nevertheless, it demonstrates the need to look beyond histomorphology for accurate diagnosis and proper management of patients, especially when the study is about a metaplastic and not dysplastic epithelium (IRANPOUR M, et al., 2021). Gonçalves JES, et al. (2017), in a review, presented several studies conducted between the years 2009 and 2017, where the use of p16 is still controversial as a prognostic marker and that it should not be used as the only parameter but in conjunction with other tests (GONÇALVES JES, et al., 2017). In our study, the use of CK7 as a biomarker sought to evaluate how much this squamous junction marker, associated with p16, might be able to present data for the prognosis of lesions. However, the results showed no significance in this association with the outcome of the women analyzed. Because this was a retrospective cross-sectional study with a loss of patients, data was lost, which is a limitation of our study. Moreover, most of the data under analysis are from fragments obtained by cervical biopsy, which may not be a faithful representation of the tissue, possibly leading to controversial diagnoses.

Currently, other studies focus on searching for additional tools to identify the promotion and progression of lesions, such as investigating genetic and epigenetic factors (POPIEL-KOPACZYK A, et al., 2023). Among the epigenetic alterations, DNA methylation, with the study of candidate genes, such as DLX4 and SIM1, is considered a prognostic marker for low-grade squamous intraepithelial lesions (SAKANE J, et al., 2015) As an alternative to cytology, comparative studies of accuracy and efficacy between double-stained cytology are considered an alternative test to cytology in screening women with HPV positive. One technique that uses double staining is CINtec® PLUS, validated for the expression of p16 and ki-67 and used as a standard of comparison to evaluate other tests (LI Y, et al., 2022).

CONCLUSION

The evaluation of clinical features and immunohistochemical markers p16 and CK7 could not show differences between patients diagnosed with typical and atypical ISM. However, our findings demonstrate that the absence of p16 and Ck7 expression is related to a favorable outcome for these patients. The accumulation of this evidence, associated with the literature, indicates the need to continue the investigation, with more specific immunohistochemical and molecular analysis, for better treatment and follow-up of women. Indicating the need for continuity of these studies to develop an effective methodology for screening patients at risk of developing the disease.

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