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PhD THESIS

NEW TRENDS IN THE DETECTION OF PRECANCEROUS LESIONS AND EARLY-STAGE CANCER OF THE UTERINE CERVIX IN PATIENTS UNDER 30 YEARS

- ABSTRACT -

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TABLE OF CONTENTS

ABSTRACT

The main risk for cervical cancer is represented by the persistent infection with HR-HPV types and types 16 and 18 are responsible for the majority of cervical cancer cases (1). Multiple studies have documented the superior sensitivity of clinically validated HR-HPV testing over Pap cytology for detecting cervical pre-cancer and cancer (2–4).

HR-HPV testing for colposcopy triage of patients with L-SIL has been proven effective in women ≥ 30 years, but is not recommended in younger women due to high positive rates of HR-HPV infection (5). Co-testing or HPV genotyping has become a standard recommendation in many international guidelines, but it is not recommended for patients under 30 years (6–10). On the other hand, due to the high number of transient HPV infections, especially in young women, the specificity of HPV genotyping as a screening method for cervical cancer is limited.

The need for a better method of triage of these cases, with ASC-US and L-SIL, opens the opportunity for immunohistochemical testing, which may provide additional elements for assessing the severity of the condition. Immunohistochemistry techniques have already been used to overstage cervical cancer samples (11) or to evaluate other gynecologic neoplasms, such as ovarian or endometrial cancer.

Combined immunohistochemical testing of p16/Ki-67 can be used to detect the onset of oncogenesis in cervical cells. P16 overexpression is caused by increased E7 oncoprotein activity (correlated with persistent HPV infection), and Ki-67 is a marker of tumor proliferation. The test is considered positive when both markers are expressed within the same cell. The combined p16/Ki-67 immunohistochemical assay can be performed on the liquid sample medium used for the Pap test and HPV genotyping, and on the biopsies. According to the latest data from the literature, combined immunohistochemical testing has a comparable sensitivity but a significantly higher specificity compared to HPV testing (12–15).

Data available in literature for the triage of ASC-US and L-SIL patients <30 years by using dual-staining is insufficient, most studies being retrospective and not exclusively addressed to this subgroup of patients. The largest study so far to have reported results of dual-staining by dividing patients based on age was performed by Bergeron et al. as part of the PALMS study. They reported an increased sensitivity of dual-staining for CIN2+ detection for patients under 30 years and significantly increased positive predictive values for CIN2+ detection compared to HPV genotyping. They also stated that dual-staining triage could considerably reduce the number of colposcopy referrals (16,17).

The fact that the same liquid-based sample can be used for cervical cytology, HPV genotyping and immunocytochemistry simplifies the procedure, by means that the patient is not obliged to pay another visit to the specialist in order to perform the test. This means that the p16/Ki-67 dual-staining could be offered as a “reflex test” for patients with cytological abnormalities on liquid-based cervical cytology analysis, or for patients with high risk HPV types detected upon HPV genotyping.

RESEARCH MOTIVATION

Although numerous retrospective studies have shown the accuracy of p16/Ki-67 dual-staining in the detection of CIN2+, more data is needed to validate the use of dual-staining p16/Ki-67 as part of a management algorithm for patients with cytological abnormalities on Pap smear.

In our studies, we aimed to update the current evidence regarding the accuracy of p16 staining and dual-staining with p16 and Ki-67 for detecting CIN2+ in the triage of patients with ASC-US or L-SIL under 30 years. Despite many data available so far regarding the improved specificity of dual-staining in young women with ASC-US or L-SIL compared to HPV genotyping, to our knowledge, this is the first prospective study conducted so far for this specific group of patients.

The prefigured results of the study aim to enrich the data available so far in literature and improve the management protocol for patients under the age of 30 years with cytological abnormalities on the Pap smear.

This research intended to answer the following questions:

• Could p16/Ki-67 dual-staining have a relevant role in the triage for biopsy of patients with ASC-US/L-SIL?

• Is p16/Ki-67 dual-staining useful in the subgroup of patients under 30 years?

• Which test or combination of tests has the highest accuracy for CIN2+ detection?

• Could p16/Ki-67 dual-staining be used in the screening of rare adenocarcinoma variants of the cervix?

The first part of the research: Personal contribution „Study of p16/ki-67 dualstaining accuracy compared to human papillomavirus testing in women with abnormal cytology under 30 years old" analyzed the utility of p16/Ki-67 in the triage for biopsy of patients with ASC-US or L-SIL.

The second part of the research: Personal contribution "Study of the role of dual-staining p16/ki-67 in the management of patients under 30 years with ASCUS/ L-SIL" analyzes the sensitivity and specificity for biopsy-confirmed CIN2+ of HR-HPV, p16/Ki-67 dual-staining, colposcopy, and combinations of the tests on all patients and separately for the ASC-US and L-SIL groups.

The third part of the research: Personal contribution "New Insights in the Diagnosis of Rare Adenocarcinoma Variants of the Cervix—Case Report and Review of Literature" represents a detailed case report of a young patient with a rare adenocarcinoma variant of the cervix, positive p16/Ki-67, negative HPV, and includes an extensive literature review on the subject.

For the first study, eligible patients who underwent colposcopy at the Department of Obstetrics and Gynecology of County Hospital Timişoara, between January 2015 and December 2016 were selected from the database.

For the second study, eligible patients that were referred for LEEP in the Department of Obstetrics and Gynecology of Timişoara University City Hospital, between January 2018 and December 2020 were selected.

Retrospective and prospective investigation was made by selection of consecutive cases based on inclusion / exclusion criteria established according to scientific objectives.

RESULTS

I. PERSONAL CONTRIBUTION: STUDY OF P16/KI-67 DUALSTAINING ACCURACY COMPARED TO HUMAN PAPILLOMAVIRUS TESTING IN WOMEN WITH ABNORMAL CYTOLOGY UNDER 30 YEARS OLD

A total of 310 patients with ASC-US or L-SIL on cervical cytology were referred for colposcopy at the Department of Obstetrics and Gynecology of County Hospital Timişoara. Patients with colposcopy negative for high-grade lesions were excluded from the study. The remaining 161 patients with ASC-US (67 patients; 42%) or L-SIL (94 patients; 58%) were referred for biopsy. Among 161 patients, 56 (35%) were <30 years and 105 patients (65%) were >30 years old. Overall, 102/161 patients (63%) tested positive for HR-HPV and 70/161 patients (43%) were positive for p16/Ki-67. CIN2-3 was detected by biopsy in 99/161 patients (61%). In ASC-US group, 38/67 patients (57%) were positive for HR-HPV, and 27/67 (40%) were positive for p16/Ki-67 test. In L-SIL group, 64/94 patients (68%) were HR-HPV-positive and 43/94 (46%) were p16/Ki-67-positive. In women over 30 years old, in ASC-US group, HR-HPV positivity rate was 63% (27/43 patients) and p16/Ki-67 positivity was 42% (18/43 patients). In L-SIL patients over 30 years, HR-HPV positivity rate was 77% (48/62 patients) and p16/ Ki- 67 positivity was 37% (23/62 patients). In women less than 30 years old, in ASC-US group, HR-HPV positivity rate was 45% (11/24 patients) and p16/Ki-67 positivity was 37% (9/24 patients). In L-SIL group less than 30 years, the positivity rate for HR-HPV test was 50% (16/32 patients) and for p16/Ki-67 dual-staining it was 62% (20/32 patients). In women with CIN2-3 detected by biopsy and <30 years old HR-HPV positivity was 50% (7/14 patients) in ASC-US and 83% (15/18 patients) in L-SIL group, p16/Ki-67 positivity rate was 57% (8/14) and 88% (16/18 patients) in the two groups respectively. The overall sensitivity and specificity of HPV genotyping for the detection of CIN2-3 were 79% and 72%, respectively in the group of patients with ASC-US, and 85% and 64%, respectively in the group of patients with L-SIL. The sensitivity and specificity rates of p16/Ki-67 dual-staining for CIN2-3, were 66% and 93%, respectively in ASC-US group, and 59% and 79%, respectively in L-SIL group. The specificity of p16/Ki-67 dual-staining was significantly increased in the group of patients <30 years old compared to patients >30 years of age ($p < 0.001$) in both ASC-US and L-SIL groups.

II. STUDY OF THE ROLE OF DUALSTAINING P16/KI-67 IN THE MANAGEMENT OF PATIENTS UNDER 30 YEARS WITH ASC-US/L-SIL

A total of 60 patients were included in the study and completed the follow up evaluation. We evaluated the correlation and distribution of p16 and Ki-67 in patients with HPV infection, HR-HPV (high-risk HPV) infection and negative HPV test and the persistence of HPV, HR-HPV infection and positive dual-staining at 6 and 12 months after LEEP. We evaluated the correlation between histological grade of low (CIN1), high-grade intraepithelial cervical lesions – CIN2+ (CIN2 and CIN3) and in situ carcinoma (CIS) with the immunohistochemical (IHC) expression of p16/Ki-67 and HR-HPV infection as presented in the flow chart. The mean age of the patients was 23.4 years. A number of 37 patients (61.6%) had L-SIL and 23 patients (38.3%) had ASC-US result on cervical cytology prior to LEEP. Regarding colposcopy, 51 patients (85%) had an abnormal result and 9 patients (15%) had normal results. The high number of abnormal colposcopies could be explained by the inclusion in our study of only patients with indication for conization. 52 patients (86.6%) had HPV infection prior to LEEP, and 36 patients (60%) had HR-HPV infection. HPV types 16 and 18 were the most frequently encountered, in 22 and respectively 15 patients. 21 patients presented a persistent HPV infection at 6 months after LEEP, out of which 20 had a HR-HPV. At 12 months after LEEP 7 patients had a persistent HPV infection, with 6 patients presenting a HR-HPV infection. HPV type 16 was the most frequently encountered in persistent infection – 7 patients presenting persistent infection at 6 months and 3 patients at 12 months after LEEP. Dual-staining for p16/Ki-67 was positive in 31 patients prior to LEEP, in 27 patients on the cervical sample specimen, in 3 patients at 6 months after LEEP and in 2 patients after 12 months. Regarding the histopathologic exam of the cervical specimen, the following results were obtained: 29 patients with CIN I, 18 patients with CIN II, 10 patients with CIN III and 2 patients with *in situ* carcinoma.

The sensitivity and specificity for biopsy-confirmed CIN2+ was crosstabulated for each test. For biopsy-confirmed CIN2+, we also analyzed the sensitivity and specificity of HR-HPV, p16/Ki-67 dual-staining, colposcopy and testing combinations on all patients, and separately for the ASC-US and L-SIL groups. HR-HPV showed a higher sensitivity in the L-SIL group (76%), but a lower specificity (50%). In the ASC-US group the specificity of HR-HPV was only 43% and the specificity was 22%. Colposcopy had a higher sensitivity compared to HR-HPV, but a very low specificity. Dual-staining had the best specificity for the ASC-US group. Combination A (HR-HPV + colposcopy) had the lowest specificity of all test combinations. Combinations B (HR-HPV + p16/Ki-67), C (colposcopy + p16/Ki-67) and D (HR-HPV + colposcopy + p16/Ki-67) presented comparable, very high specificity; however, the sensitivity was lower for combinations B and D compared to combination C. p16/Ki-67 alone or in combination with colposcopy and/or HR-HPV improved the overall specificity for CIN2+ detection. Resection margins

were negative (in healthy tissue) for all patients and no further surgical treatment was performed during the 12 months follow up period. Cervical cytology revealed to be normal (NILM) for all patients at the 6 and 12 months follow up after LEEP. For the patients with persistent HR-HPV infection and/or positive dual-staining at 6 and respectively 12 months after LEEP we recommended a follow up at 3 months interval by co-testing (i.e. cervical cytology and HR-HPV detection). No further treatment was applied up to date.

III. PERSONAL CONTRIBUTION: NEW INSIGHTS IN THE DIAGNOSIS OF RARE ADENOCARCINOMA VARIANTS OF THE CERVIX – CASE REPORT AND REVIEW OF LITERATURE

We reported the case of a 29-year-old patient with low-grade squamous intraepithelial lesion (L-SIL), negative human papilloma virus (HPV), positive p16/Ki-67 dual-staining and colposcopy suggestive for severe dysplastic lesion. The patient underwent a loop electrosurgical excision procedure (LEEP), the pathology report revealing mesonephric hyperplasia and adenocarcinoma. The patient also opted for non-standard fertility-sparing treatment. The trachelectomy pathology report described a zone of hyperplasia at the limit of resection towards the uterine isthmus. Two supplementary interpretations of the slides and immunohistochemistry (IHC) were performed. The results supported the diagnosis of mesonephric adenocarcinoma, although with difficulty in differentiating it from mesonephric hyperplasia. Given the discordant pathology results that were inconclusive in establishing a precise diagnosis of the lesion and the state of the limits of resection, the patient was referred to a specialist abroad. Furthermore, the additional interpretation of the slides and IHC were performed, the results suggesting a clear cell carcinoma. The positive p16/Ki-67 dual-staining prior to LEEP, the non-specific IHC and the difficulties in establishing a diagnosis made the case interesting.

GENERAL CONCLUSIONS AND ORIGINAL CONTRIBUTIONS

In our first study, we obtained similar results to those previously reported in retrospective studies. The dual-staining test performed significantly better in terms of specificity in the group of patients under 30 years. For our first retrospective study we concluded that dual-staining p16/Ki-67 alone or in combination with HR-HPV and/or colposcopy showed a higher specificity that HR-HPV and/or colposcopy for the diagnosis of biopsy confirmed CIN2+ in patients under 30 years. Colposcopy+p16/Ki-67 and HR-HPV+ colposcopy+p16/Ki-67 showed the highest specificity in our study. We consider p16/Ki-67 could be useful in the triage of young patients with ASC-US or L-SIL and should be taken into consideration for the diagnostic algorithm of this subgroup of patients.

The results of our first study, conducted in a retrospective manner, encouraged us to perform a second, prospective study for patients under 30 years in order to quantify the role of p16/Ki-67 dual-staining and HPV genotyping in the detection of high-grade cervical lesions in patients with ASC-US or L-SIL on cervical cytology.

In our second study we evaluated the correlation and distribution of p16 and Ki-67 in patients with HPV infection, HR-HPV (high-risk HPV) infection and negative HPV test and the persistence of HPV, HR-HPV infection and positive dual-staining at 6 and 12 months after LEEP. Also, we evaluated the correlation between histological grade of low (CIN1), high-grade intraepithelial cervical lesions – CIN2+ (CIN2 and CIN3) and in situ carcinoma (CIS) with the immunohistochemical (IHC) expression of p16/Ki-67 and HR-HPV infection.

The different age distribution has an important impact on the rate of HR-HPV positive patients. In our study the mean age of the patients was 23.4 years.

A very high prevalence of HPV infection has been reported in literature in LSIL patients (86-97%) and ASC-US (89.5%). HPV infection has also been detected in 27% of patients with NLM results (18). HR-HPV infection has been reported to be more common in women under 30 years with abnormal cytology, compared to older women (14,19). In our study we had the following distribution of HPV infection: 52 patients (86.6%) had HPV infection prior to LEEP, and 36 patients (60%) had HR-HPV infection. 33 out of 37 patients from the L-SIL group had HPV infection prior to LEEP, and 23 had HR-HPV strains. Regarding the ASC-US group, 19 out of 23 patients had HPV infection prior to LEEP, out of which 13 had HR-HPV.

We managed to compare the performance of p16/Ki-67 dual-staining and HPV genotyping in the detection of high-grade cervical lesions in patients with ASC-US or L-SIL on cervical cytology (Pap smear), to quantify the performance of p16/Ki-67 dual-staining in the detection of CIN2+ in patients with ASC-US or L-SIL on cervical cytology, to analyze the distribution and expression of p16/Ki-67 in patients with HRHPV infection (single strain or co-infection), to evaluate the persistence of positive p16/Ki-67 and HR-HPV after LEEP. We also evaluated the performance for CIN2+ detection of each individual test and the best combination of tests and the impact of age distribution on the rate of HR-HPV positive and p16/Ki-67 positive patients.

Despite many data available up to now regarding the improved specificity of dual-staining in young women with ASC-US or L-SIL compared to HPV genotyping, to our knowledge, our second study represents the first prospective study conducted so far for this specific group of patients.

Furthermore, we also compared the accuracy of dual-staining p16/Ki-67 combined with HPV genotyping and colposcopy in order to identity the combination of tests that offers the best accuracy for CIN2+ prediction.

The weakness of our studies is the relative small number of cases involved. More data is necessary in order to validate the use of the dual-staining p16/Ki-67 as part of a management algorithm for patients with cytological abnormalities on Pap smear.

We also identified and reported a rare case of non-HPV adenocarcinoma variant of cervical cancer with positive dual-staining prior to LEEP. Cytological diagnosis is difficult in differentiating hyperplasia or inflammation from malignant cells in the majority of cases and discordant immunohistochemistry results between laboratories can be frequently encountered in clinical practice (20–22). These findings suggest caution in the initial diagnosis through immunostaining. We believe it is important to recognize these unique variants of cervical adenocarcinoma at an early stage, as they can associate a poor clinical outcome given the usually advanced stage at the time of diagnosis. Also, the positive dual-staining opens the window of opportunity for further use of p16/Ki-67 for the diagnosis of rare non-HPV cervical cancer variants.

In our studies, dual staining p16/Ki-67 showed superior specificity compared to the HPV genotyping test, especially in the group of patients under 30 years. Our data indicates that dual staining p16/Ki-67 might be an option in the triage of patients younger than 30 years with ASC-US or L-SIL on cytology test, prior to performing colposcopy and biopsy. Therefore, the major benefit of using dual staining p16/Ki-67 as a triage for colposcopy could considerably reduce the number of colposcopy referrals and reduce the impact of overtreatment of precursor lesions.

We believe our studies represent an important contribution in the field, with the novelty of having performed the first prospective study for patients under 30 years for the evaluation of p16/Ki-67 dual-staining in the triage of patients with ASC-US/LSIL.

Our results suggest dual-staing should be taken into consideration in the management algorithm of this specific subgroup of patients.

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