Dunaevskaya V. V., Med V. V., Krotevych M. S. Immunohistochemistry in pre-invasive vulvar lesions: observations, concerns and an algorithmic approach. Journal of Education, Health and Sport. 2024;53: 256-269. eISSN 2391-8306.
https://dx.doi.org/10.12775/JEHS.2024.53.021
https://apcz.umk.pl/JEHS/article/view/53044
https://zenodo.org/records/12635716

The journal has had 40 points in Ministry of Education and Science of Poland parametric evaluation. Annex to the announcement of the Minister of Education and Science of 05.01.2024 No. 32318. Has a Journal's Unique Identifier: 201159. Scientific disciplines assigned: Physical culture sciences (Field of medical and health sciences); Health Sciences (Field of medical and health sciences). Punkty Ministerialne z 2019 - aktualny rok 40 punktów. Załącznik do komunikatu Ministra Edukacji i Nauki z dnia 05.01.2024 Lp. 32318. Posiada Unikatowy Identyfikator Czasopisma: 201159. Przypisane dyscypliny naukowe: Nauki o kulturze fizycznej (Dziedzina nauk medycznych i nauk o zdrowiu); Nauki o zdrowiu (Dziedzina nauk medycznych i nauk o zdrowiu).

The authors declare that there is no conflict of interests regarding the publication of this paper.
Received: 06.01.2024. Revised: 19.01.2024. Accepted: 27.01.2024. Published: 28.02.2024.

IMMUNOHISTOCHEMISTRY IN PRE-INVASIVE VULVAR LESIONS: OBSERVATIONS, CONCERNS AND AN ALGORITHMIC APPROACH

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Abstract

The aim of the study was to investigate the presence of immunohistochemical markers p16, p53 and Ki67 and their diagnostic and prognostic value in women with pre-invasive vulvar lesions such as vulvar high-grade squamous intraepithelial lesions (VHSIL) and differentiated vulvar intraepithelial neoplasia (dVIN). **Materials and methods.** The results of immunohistochemical (IHC) examination of samples obtained from 253 women diagnosed with HSIL or dVIN who asked for medical care at the National Cancer Institute (Kyiv, Ukraine) in 2017-2023 were analysed. 155 of all women examined were diagnosed with dVIN and 98 with VHSIL. All patients underwent a vulvar biopsy. Histological typing of biopsy samples was performed using routine haematoxylin and eosin staining and immunohistochemical (IHC) examination. The IHC study was performed using monoclonal mouse antibody p16 (Monoclonal

Mouse Antibody p16 (Mob575-01)) using the Thermo scientific PA1-16662 system, monoclonal mouse antibody Ki-67 (Monoclonal Mouse Anti-Human Ki-67 Antigen Clone MIB-1 (Dako IR-626)) and monoclonal mouse antibody p53 (Monoclonal Mouse Anti-Human p53 Protein Clone DO-7 (Dako IS-616)) using the EnVisionTM FLEX detection system (Dako, Denmark). **Results.**The average age of patients with dVIN was 58.26±10.17 years old, and the average age of patients with VHSIL was 39.65±10.22 years old. According to the results of the study, all patients with VHSIL (n=98) had positive staining for Ki67 in the middle and upper epithelial sections, and only 72% (n=71) - for p16. Staining for p53 was negative in all cases. That is, 28% (n=27) of the patients with VHSIL were negative for p16 and positive for Ki67. p53 staining showed the presence of a "wild-type" variant in 65 patients with dVIN (42%) and a "mutant" variant in 90 women (58%). None of the patients showed positive staining for p16. As for VHSIL, the Ki67 marker was detected in 100% of cases and in all situations in the middle and upper parts of the epithelium, while with dVIN patients, such staining was observed in only 45 women (29%). **Conclusions.** The determination of IHC markers p16, p53 and Ki67 allows to distinguish dVIN from VHSIL, especially in difficult diagnostic cases. In addition, the presence of mutant p53 indicates the possibility of rapid progression to cancer and requires immediate and more aggressive treatment and follow-up.

Key words: differentiated vulvar intraepithelial neoplasia; high grade squamous intraepithelial lesion of the vulva; immunohistochemical markers; p16; Ki67; p53

According to significant international studies, the prevalence of vulvar intraepithelial neoplasia (VIN) has recently increased, especially in young women [1, 2, 3]. Therefore, special attention is drawn to such pre-tumour conditions as human papillomavirus (HPV)-dependent vulvar high-grade squamous intraepithelial neoplasia (VHSIL) and differentiated vulvar intraepithelial dysplasia (dVIN), which have a fairly high probability of malignancy (5.7% and 32.8%, respectively) [4-7].

Recent decade's research has been focused on finding diagnostic and prognostic markers of atypia. For example, p53 is a tumour suppressor gene involved in maintaining genomic integrity by controlling cell cycle progression or inducing apoptosis. About 50% of primary human cancers carry mutations in this gene [8]. The tumour-suppressive activity of p53 is attributed to its ability to regulate the transcription of many different genes in response to a number of stress signals [9]. Some viral oncogenes, such as the HPV E6 viral oncogene, lead to functional inactivity of p53, causing deregulation of the expression of many genes regulated by p53, such as those involved in apoptosis, DNA stability and cell proliferation [10].

Many tumours are characterised by the inactivation of the p16INK4a gene, which leads to dysregulation of the cell cycle and uncontrolled cell proliferation. In tumours associated with the transformational effect of HPV, there is an increase in the expression of p16INK4a, which is ineffective in regulating the cell cycle [11-13]. The expression of the cyclin-dependent kinase inhibitor p16INK4A (p16) is closely correlated with the presence of high-risk HPV types, and p16 overexpression is considered a surrogate marker for HPV-induced neoplasia [14, 15]. The rise of p16 protein production is mainly associated with increased transcription mediated by the HPV-encoded oncoprotein E7. The latter functionally inactivates retinoblastoma protein (RB), releasing p16 from negative feedback control [16].

The scientific literature reports the positivity of p16-IHC for VHSIL [17, 18]. The combination of a positive HPV polymerase chain reaction (HPV-PCR) and a block-positive p16-IHC is considered by some authors to be the "gold standard" for the diagnosis of VHSIL [19]. Determination of p16-IHC can also help distinguish VHSIL from benign changes such as squamous cell hyperplasia, radiation changes, and transitional zone mucosa, which are p16-negative [20]. At the same time, complete negativity to minimal focal p16-IHC staining is reported for dVIN. In the case of dVIN that histologically imitates VHSIL, negative p16-IHC helps to distinguish it from VHSIL [21].

Ki-67 is a nuclear antigen present in proliferating human cells at all stages of the cell cycle except G0. MIB-1 is a monoclonal antibody against Ki-67. In normal vulvar epithelium, MIB-1 stains mainly the parabasal layers and rarely the basal layers. In VHSIL, increased MIB-1 staining can be seen in both the basal and parabasal layers, with spread to the upper two-thirds of the epithelium. Increased MIB-1 expression is also observed in the basal and parabasal layers in dVIN. This may help distinguish dVIN from lichen sclerosus, which usually shows only basal MIB-1 expression [21].

According to the most recent consensus on pre-invasive vulvar lesions, a panel of ICH markers p53, p16, and ki-67 helps to distinguish VVHSIL from dVIN [22]. Mutant patterns of p53 expression are used to confirm the histological diagnosis of dVIN, as they reportedly reflect the TP53 mutations that characterise dVIN [23-25]. However, in 17-42% of dVINs, wild-type p53 expression can be detected, which is usually observed in non-dysplastic lesions [26-29].

At the same time, the prognostic value of p16 and p53 expression in patients with squamous cell carcinoma of the vulva is controversial. Some authors have suggested that these markers are not independent prognostic factors [30-33], while others have postulated that surgical tactics may be altered depending on the presence or absence of HPV DNA and/or p16 immunohistochemistry [34, 35].

Thus, **the aim** of our study was to investigate the presence of immunohistochemical markers p16, p53 and Ki67 and their diagnostic and prognostic value in women with pre-invasive vulvar lesions such as vulvar high-grade squamous intraepithelial lesions and differentiated vulvar intraepithelial neoplasia.

Materials and methods of the study

To achieve this goal, we analysed the results of IHC examination of samples obtained from 253 women aged 25 to 70 years old diagnosed with VHSIL or dVIN who sought medical care at the National Cancer Institute (Kyiv, Ukraine) in 2017-2023 years. Patients were included in the study after obtaining written informed consent in accordance with the principles of the Helsinki Declaration of Human Rights, the Council of Europe Convention on Human Rights and Biomedicine and the relevant laws of Ukraine. The diagnosis was made on the basis of medical history, complaints, clinical examination, vulvoscopy, dermoscopy, vulvar biopsy and histological examination of pathologically altered tissues. The criteria for inclusion of patients in the study for treatment were morphological confirmation of the diagnosis, absence of severe comorbidities and written consent to treatment.

Of all the women examined, 155 had dVIN and 98 had VHSIL. The age of patients with dVIN ranged from 25 to 70 years old and averaged 58.26±10.17 years old, while the age of VHSIL patients examined was from 25 to 66 years old, and the average age was 39.65±10.22 years old.

All patients underwent a vulvar biopsy. Histological typing of biopsy samples was performed using routine haematoxylin and eosin staining and immunohistochemical (IHC) examination. The vulvar biopsy material in cassettes was placed for 16 hours in a container for fixation in buffered 10% formalin, pH=7.4. This material was sealed in paraffin using a Histos-5 histoprocessor (Milestone, Italy), according to the programme for surgical material - 4 mm. After completion of the paraffin embedding programme, the cassettes were removed from the paraffin block of the histoprocessor and, using the HESTION TEC-2800 Embedding Centre, the tissue pieces were filled with molten paraffin into moulds, followed by solidification in the refrigeration module of the HESTION TEC-2800 Cryo Console. Histological sections of 5 μm thickness were made from the obtained paraffin blocks using a Microm HM325 microtome (Thermo Scientific, Germany). Sections were stained with haematoxylin and eosin for pathological examination of the tumour, morphometry and calculation of the size of viable tumour tissue.

The IHC study was performed using monoclonal mouse antibody p16 (Monoclonal Mouse Antibody p16 (Mob575-01)) using the Thermo scientific PA1-16662 system, monoclonal mouse antibody Ki-67 (Monoclonal Mouse Anti-Human Ki-67 Antigen Clone MIB-1 (Dako IR-

626)) and monoclonal mouse antibody p53 (Monoclonal Mouse Anti-Human p53 Protein Clone DO-7 (Dako IS-616)) using the EnVisionTM FLEX detection system (Dako, Denmark). Antigen demasking was performed in citrate buffer with pH=6.0 at 95°C. Primary antibodies were incubated at room temperature for 30 min and secondary antibodies for 20 min. The sections were counterstained with Gill's haematoxylin. For positive control, tissue samples with positive reactivity were used, and for negative control, the procedure was performed without primary antibodies. The resulting samples were analysed by CellSens software under standardised conditions.

The positive results were characterised by nuclear staining for p53, Ki-67 and p16. The IHC biomarkers selected are commonly used for the diagnosis and recognition of VHSIL and dVIN by the nature of basal layer atypia [21,36]. Intense p53 staining of almost all tumour cell nuclei indicates a p53 mutation. A variable, heterogeneous positive staining pattern was interpreted as the presence of a "wild-type" variant. For p16, the positive pattern was a block-like positive nuclear pattern with ± cytoplasmic staining of almost all tumour cells. Variable and/or partial positive staining was interpreted as negative.

Statistical processing and analysis of data was carried out using Statistica 7.0 for Windows and Microsoft Excel. Standard methods of descriptive and comparative analysis were used in the study. The mean value (M) and standard deviation (±SD) were calculated. The reliability of the parametric values was assessed by the Student's test. A value of $p<0.05$ was considered statistically significant.

Results of the study and their discussion

The mean age of patients with dVIN in our study was 58.26±10.17 years old, and with VHSIL - 39.65±10.22 years old ($p<0.05$). The majority (87%, n=85) of women with VHSIL were under the age of 50, whereas with dVIN , 90% (n=140) were over 50 years old. These findings are consistent with the data that there are two subtypes of squamous cell carcinoma of the vulva. The more common type is usually found in older women and is usually associated with lichen sclerosus and/or dVIN [37], and is often associated with tumour suppressor gene p53 mutations [38,39]. The other subtype is more common in younger women and is mainly associated with HPV [40,41].

According to the results of our study, all patients with VHSIL (n=98) were positive for Ki67 in the middle and upper epithelial layers, and only two thirds (n=71, 72%) were positive for p16. Staining for p53 was negative in all cases. In other words, 28% (n=27) of patients with VHSIL were negative for p16 and positive for Ki67.

Other studies have shown similar results. For example, N. B. Thuijs and colleagues reported that VHSIL showed diffuse staining for p16INK4a in two-thirds or more of the

epithelium, with the majority of VHSIL (88.9%) showing completely diffuse staining for p16INK4a and increased proliferative activity in two-thirds or more of the epithelium as measured by Ki-67 expression [42].

Staining for p53 showed the presence of the "wild" variant in 65 patients with dVIN (42%) and the "mutant" variant in 90 women (58%). None of the patients had positive staining for p16. It should be noted that while in VHSIL cases, the Ki67 marker was detected in 100% of cases and in all situations in the middle and upper epithelium, in dVIN cases, such staining was observed in only 45 patients (29%). We have obtained three variants of the combination of IHC markers p16 and Ki67: p53 "wild-type" and Ki67 (in basal and suprabasal keratinocytes); p53 "mutant" and Ki67 (in basal and suprabasal keratinocytes) and p53 "mutant" and Ki67 (in the middle and upper parts of the epithelium). The first variant was registered in 65 women (42%), the second and third in 45 patients (29%).

According to various studies the prevalence of p53 expression in squamous cell carcinoma of the vulva is 28-78% [43-49]. VHSIL usually shows wild-type p53-IHC staining, characterised by sporadic nuclear staining, with a weakly positive or completely negative basal epithelial layer. Some VHSILs show weak patchy staining in the basal layer and a higher proportion of positive nuclei in the suprabasal layers [50]. dVIN more often demonstrates a wild-type p53 staining pattern. However, according to some researchers, p53-IHC has limitations in distinguishing dVIN from lichenoid conditions: increased p53 staining can be observed in 5-61% of cases of sclerosing lichen and up to 40% of cases of squamous hyperplasia due to oxidative stress. Moreover, p53 positivity has also been observed in "normal" vulvar skin [21,51].

Some researchers have pointed to an inverse correlation between p53 expression and p16 expression, and that p53 gene mutation is mainly observed in vulvar cancer that is not associated with HPV infection [43,47,52].

In our study, 28% of women with VHSIL did not have p16. This may be due to an incorrect histological diagnosis or other reasons. Thus, in the study by L. Barlow et al., 10% of squamous cell carcinomas of the vulva did not have p16/HPV DNA or p53 expression, and they postulated that the mechanism of carcinogenesis in this group is unknown [43]. Similar results were obtained by some other authors [53-55]. L. S. Nooij and colleagues identified a third molecular subtype of vulvar cancer that was HPV and p53 negative but had wild-type p53 with frequent NOTCH1 mutations [35]. T. Lozar et al. suggested that p16-negative squamous intraepithelial lesions (SIL) of the vulva may be similar to cutaneous squamous cell lesions, such as actinic keratosis, associated with cutaneous HPV types (e.g., β-HPV) [56]. They detected β-HPV in 70% of p16-negative patients and concluded that there was an association between p16-negative SIL and the presence of β-HPV types, which they suggested was an alternative

etiological pathway that could be involved in the initiation of carcinogenesis. In addition, recent studies have also identified a new precursor to HPV-independent squamous cell carcinoma of the vulva, called differentiated exophytic intraepithelial lesions of the vulva (DEVIL). DEVIL are acanthotic lesions without sufficient histological atypia to diagnose dVIN and demonstrate wild-type p53 expression on IHC examination [53,57,58]. The authors believe that there is a need for IHC markers that can facilitate the diagnosis of HPV-independent precursors, particularly in lesions with wild-type p53 [24,59].

Conclusions

The determination of IHC markers p16, p53 and Ki67 allows to distinguish dVIN from VHSIL in most cases, especially in difficult diagnostic cases. In addition, the presence of mutant p53 indicates the possibility of rapid progression to cancer and requires prompt and more aggressive treatment and follow-up.

Author's contribution: the article is written independently.

Funding: This study received no external finding.

Institutional Review Board Statement: A positive of the boethics commission was received for conducting the research State Institution "Institute of Pediatrics, Obstetrics and Gynecology of the NAMS of Ukraine" (protocol No 1 dated December 20, 2019), the main moral and ethical principles of the Helsinki Declaration of the world Medical Assotiaton for Biomedical Research are observed.

Informed Consent Statement: Written informed consent for treatment, use of the patients' personal data and their use was obtained from all examined women.

Data Availability Statement: All information is publicly available, data on a specific patient can be obtained upon request from the author.

Conflict of Interest: The author no conflict of interest.

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