ONCOLOGY

PRECURSORS, PATHWAYS OF CARCINOGENESIS AND MOLECULAR MARKERS OF VULVAR SQUAMOUS CELL CARCINOMA. LITERATURE REVIEW

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ABSTRACT

The review analyzes and summarizes the results of the studies on the pathogenesis of vulvar squamous cell carcinoma and its diagnostic features, reviews precursors and molecular subtypes of carcinomas. Despite the relatively low incidence of this tumor, over the past few decades, there has been an upward trend in its incidence, including the incidence among young women. According to the latest World Health Organization classification of lower genital tumors from 2020, vulvar squamous cell carcinoma is divided into human papillomavirus (HPV) associated and HPV-independent. While these carcinomas are often morphologically similar, their mechanisms of carcinogenesis, precursors, and clinical outcomes are different. Just the detection of virus DNA in a tumor is not enough to establish HPV status. Meanwhile immunohistochemical detection of the expression of p16 and p53 proteins allows not only to separate two pathogenetic pathways of carcinogenesis, but also to identify its molecular subtypes. The data on the possible use of p16 and p53 expression as the disease prognosis molecular markers have been obtained. Currently, the tactics of treatment and monitoring patients does not depend on the HPV status of carcinoma; however, the results of recent studies suggest that women with HPV positive vulvar cancer have significantly higher survival rates and a lower risk of recurrence. Understanding the mechanisms of carcinogenesis and improving its diagnosis will advance the assessment of the individual risk of the progression of precancerous lesions, as well as the outcome and the occurrence of tumor recurrence.

Key words: *vulvar squamous cell carcinoma, intraepithelial neoplasia, human papillomavirus, p16, p53*

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ПРЕДШЕСТВЕННИКИ, ПУТИ КАНЦЕРОГЕНЕЗА И МОЛЕКУЛЯРНЫЕ МАРКЕРЫ ПЛОСКОКЛЕТОЧНОЙ КАРЦИНОМЫ ВУЛЬВЫ. ЛИТЕРАТУРНЫЙ ОБЗОР

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РЕЗЮМЕ

В обзоре проанализированы и обобщены результаты исследований, изучающих патогенез плоскоклеточного рака вульвы и особенности его диагностики, рассмотрены предшествующие состояния и молекулярные подтипы карцином. Несмотря на относительно низкую встречаемость опухоли, за последние несколько десятилетий отмечается тенденция к росту заболеваемости, в том числе среди молодых женщин. Согласно последней классификации Всемирной организации здравоохранения опухолей нижнего отдела генитального тракта от 2020 г., плоскоклеточный рак вульвы подразделяется на ассоциированный с вирусом папилломы человека (ВПЧ) и ВПЧ-независимый. В то время как морфологически эти карциномы часто являются сходными, механизмы канцерогенеза, предшественники, а также клинические исходы у них различны. Только лишь обнаружение ДНК вируса в опухоли недостаточно для установки ВПЧ-статуса. В то же время иммуногистохимическое обнаружение экспрессии белков p16 и p53 позволяет не только разделить два патогенетических пути канцерогенеза, но и выявить его молекулярные подтипы. Получены данные о том, что экспрессию p16 и p53 возможно также использовать в качестве молекулярных маркеров прогноза заболевания. В настоящее время тактика лечения и наблюдения пациенток не зависит от ВПЧ-статуса карциномы, однако результаты исследований последних лет говорят о том, что женщины с ВПЧ-положительным раком вульвы имеют значительно более высокие показатели выживаемости и меньший риск возникновения рецидивов. Понимание механизмов канцерогенеза и усовершенствование его диагностики позволит улучшить оценку индивидуального риска прогресса предопухолевых состояний, а также исход и возникновение рецидива опухоли.

Ключевые слова: *плоскоклеточная карцинома вульвы, интраэпителиальная неоплазия, вирус папилломы человека, p16, p53*

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INTRODUCTION

Despite the fact that vulvar squamous cell carcinoma (VSCC) is a rare malignant tumor. In the last decade, the attention of an increasing number of scientists has been focused on studying its pathogenetic mechanisms, molecular events of carcinogenesis, as well as searching for reliable prognostic biomarkers of the disease [1–8]. In 2020, The World Health Organization has published a new classification of lower genital tumors, according to which vulvar squamous cell carcinoma (VSCC) are divided into human papillomavirus (HPV) associated and HPV-independent [9]. Unlike cervical squamous cell carcinoma, which in most cases is associated with HPV of high carcinogenic risk (HCR) [10–12], vulvar squamous cell carcinoma is more often HPV-independent; its molecular mechanisms are still poorly studied [13, 14]. Currently, the tactics of treatment and monitoring patients with an established diagnosis of VSCC depends only on the stage of the disease and somatic status [15, 16]. However, the results of large studies in recent years suggest that women with HPV-positive vulvar squamous cell carcinoma have significantly higher overall survival rates and a lower risk of recurrence [2, 6]. At the same time, a number of authors in their works do not come to the conclusion about the differences in prognosis depending on the mechanism of pathogenesis [17–19]. Differences in the results of studies may be due to the lack of a standardized set of methods for diagnosing HPV status. Despite the high sensitivity of the polymerase chain reaction (PCR) method, its results cannot be interpreted as sufficient, since only the detection of HPV DNA HCR is not a proof of its role in the process of malignancy [20, 21]. According to recent studies, immunohistochemical (IHC) detection of the expression of p16 and p53 proteins allows not only to separate the two pathogenetic pathways of carcinogenesis of the external genital organs [22–24], but also to use them as prognostic markers [5, 7, 25]. Thus, understanding the pathogenetic mechanisms, improving diagnostic methods, as well as determining the disease prognosis justify the need for a detailed and further study of the issue.

THE AIM OF THE REVIEW

To characterize the similarities and differences between HPV-associated and HPV-independent vulvar squamous cell carcinoma; to consider the probable and currently known carcinogenesis pathways, precursors, as well as the features of their diagnosis.

MOLECULAR MECHANISMS OF PATHOGENESIS OF TWO TYPES OF CARCINOMAS

HPV-associated carcinomas. Human papillomavirus is the most common sexually transmitted infection [12, 26]. However, in most cases it is transient, and subsequently the virus is eliminated from the body without transforming the cells [27]. Viral integration into the cell genome is an important event of carcinogenesis, leading to increased expression of the main viral E6 and E7 proteins, which in turn inactivate two tumor suppressors, namely p53 and retinoblastoma protein (pRb) [14, 27, 28]. E6 forms a triple complex with E6-associated protein (E6AP) and p53, altering the functional ability of p53 and causing degradation of this cellular protein through the ubiquitin-mediated proteolysis pathway, which leads to the absence of cell cycle arrest [29, 30]. E7 binds to pRb, which leads to the labeling of pRb for degradation, thereby releasing the E2F transcription factor from the pRb-E2F complex, which regulates cell proliferation, resulting in uncontrolled cell division [29, 31]. The Rb/E2F complex inhibits the transcription of several genes, including the *CDKN2A* gene encoding p16INK4a (p16). Consequently, the cleavage of Rb family members by the E7 protein leads to overexpression of p16 due to the release of the *CDKN2A* gene [14, 27]. An increase in the expression of the p16 protein occurs as an attempt to control uncontrolled cell division, which is mediated by a violation of the pRb pathway. However, the transduction of signals for the cell cycle arrest is also counteracted by E7-mediated activation of A and E cyclins [32]. Accelerated cell proliferation caused by high-risk HPV oncoproteins contributes to the accumulation of genetic defects, such as deletions, amplifications, translocations and chromosomal rearrangements, and leads to cell mutation [29].

HPV-independent carcinomas. In contrast to the relatively large amount of data on the pathogenesis of HPV-associated tumors, the HPV-independent pathway of VSCC has been studied much poorly, and the molecular mechanisms involved in the development of such VSCC have not yet been fully elucidated [14, 28]. According to studies, carcinomas etiologically unrelated to HPV more often contain mutations in the TP53 tumor suppressor gene. The product of this gene is the p53 protein (p53wt), whose function is to prevent DNA replication and trigger apoptosis in cells with abnormal DNA. This preserves the stability of the genome and prevents mutational changes [1, 7, 33]. Missense mutations in *TP53* lead to the accumulation of mutant p53 protein (p53mut) in cell nuclei, resistant to degradation and unable to perform its functions [7, 34]. According to studies, from 67 to 80 % of all HPV-independent tumors had mutations in this gene [1, 25, 35, 36]. At the same time, they are considered an early event of a carcinogen, since they were also detected in previous lesions [28, 37]. It is worth noting that mutations in the *TP53* gene can also occur in HPV-associated carcinomas, although much less frequently (about 25 % of cases). However, it is more likely that these changes are associated with the tumor progression, and not with the initiation of the process [29, 33].

Studies conducted using new generation sequencing methods have shown that *CDKN2A* is the second most frequently mutated gene, accounting for about 30–40 % of all genetic changes. As a result of its mutation, the p16 encoded by it is inactivated, which leads to disruption

of the cell cycle [18, 29, 33]. It is assumed that the combination of mutations in the *TP53* and *CDKN2A* genes correlates with a significantly worse prognosis of the disease [1, 38]. Also, *HRAS*, *PIK3CA*, *PTEN* and *NOTCH1* mutations were detected in HPV-independent carcinogenesis [8, 29, 39]. While some studies suggest that HPV-independent tumors have a large mutational load [25, 40], others [13, 39] indicated that the mutational load does not significantly depend on the HPV status. Perhaps this is due to differences in the methods of establishing the HPV status of the tumor and the use of different molecular markers.

In 2017, L.S. Nooij et al. in their work confirmed the third molecular subtype of VSCC independent of HPV and *TP53* mutation using genomic sequencing [25]. The group included 43 samples from a group of 236 subjects (18 %) and showed the highest frequency of *NOTCH1* and *HRAS* mutations. *NOTCH1* is a transmembrane receptor involved in cell differentiation and proliferation. The *HRAS* gene is involved in the PIK3CA/AKT/mTOR pathway, which regulates the processes of cell division and apoptosis. Their mutations can probably lead to the transduction of signals that activate cell proliferation and inhibit cell death. The mechanism of carcinogenesis in this group is difficult to understand and requires further study [22, 24]. Later, other authors also identified this subtype of tumors, but with different frequency of occurrence – from 4 % [41] and 10 % [22] to 25.7 % [23]. In the study by K.E. Kortekaas et al. 63 (15 %) cases from 413 studied samples were not associated with HPV and *TP53* mutation [5], while L. Woelber et al. obtained this subtype in 116 of 411 carcinomas (28 %) [24].

PREVIOUS CONDITIONS AND MORPHOLOGICAL FEATURES

Vulvar intraepithelial neoplasia. The development of invasive carcinoma is a slow, multi-stage process that many years [14]. Vulvar intraepithelial neoplasia (VIN) is a non-invasive squamous cell lesion and one of the most common precursors of vulvar squamous cell carcinoma. HPV-associated carcinomas usually develop from a high-grade squamous intraepithelial lesion (HSIL), previously known as usual vulvar intraepithelial neoplasia (uVIN), whereas HPV-independent arise from a precancerous lesion called differentiated VIN (dVIN) [42, 43].

According to studies, the detection rate of HSIL is significantly higher than dVIN (90–95 % vs. 5–10 %, respectively), but the probability of progression to invasive carcinoma is higher in the group of patients with diagnosed differentiated neoplasia. The risk of malignant transformation of the vulva is up to 15 % in women with unattended HSIL, below 5 % for HSIL after treatment and approximately 30 % for patients with dVIN [44, 45]. In addition, dVIN is associated with a shorter progression period. In the study of S.M. Bigby et al. the mean interval between dVIN biopsy and diagnosis of squamous cell carcinoma was 43.5 months (range 8–102 months) [46]. Similar indicators were shown in another study that included 1,826 women with HSIL and 67 women with dVIN; the latter had a higher risk of progression to malignant neoplasm (32.8 % vs. 5.7 %, respectively), which occurred on average after 22.8 months compared with 41.4 months for HSIL [47].

Morphological changes in HSIL are similar to all HPV-associated intraepithelial lesions, for example, as in high-grade cervical dysplasia. Dysplastic changes affect usually the entire thickness of the epithelium, are characterized by pronounced cellular polymorphism, as well as a violation of the nuclear-cytoplasmic ratio with nuclear hyperchromia. Koilocytic changes in the upper layers of the epithelium are characteristic [45]. Morphological diagnosis of dVIN presents certain difficulties due to indolent atypia, a high degree of differentiation of dysplastic cells, as well as due to the lack of accurate and reproducible diagnostic evaluation criteria. Para- and dyskeratosis, basal nuclear atypia of cells with an increase in size and nucleoli are characteristic. Koilocytic changes are not detected [44, 48]. However, there is evidence that some HPV-associated precursors mimic HPV-independent lesions, and, conversely, some HPV-independent intraepithelial precursors may have HSIL traits [25, 49].

Lichen sclerosus. Chronic inflammatory disease of autoimmune etiology, characterized morphologically by dermal lymphoid infiltration, collagen hyalinization and hyperkeratosis. Previously, lichen sclerosus (LS) was designated by the following terms: kraurosis, leukoplakia, sclerotic lichen or atrophic lichen [45]. It is assumed that the mechanism of occurrence of LS is the effect of activated T cells releasing interleukin-4 and transforming growth factor β on the cells of the basal layer. Thus, these cytokines activate fibroblasts, which leads to fibrosis. A long-term chronic process leads to the accumulation of genetic mutations [50, 51]. According to M.D. Trietsch et al., the frequency of *TP53* mutations in LS is 6 % [1]. In a study by L. Micheletti et al. among 976 women with LS, 34 (3.5 %) patients subsequently developed intraepithelial neoplasia, and 26 (2.7 %) had invasive squamous cell carcinoma [52]. Retrospective cohort studies show that LS is detected in the adjacent tissue from 5 to 88 % of invasive carcinomas, more often in combination with dVIN [4, 46, 53]. According to a recently published systematic review, the risk of developing VSCC in women with LS ranges from 3 to 21.8 %. Such variation is probably due to the wide variation in the occurrence of LS in studies, as well as diagnostic difficulties in differentiating LS from dVIN [54].

Vulvar aberrant maturation. A new general term for HPV-independent lesions combining vulvar aberrant maturation (VAM) with minimal nuclear atypia. It includes differentiated exophytic vulvar intraepithelial lesion (DEVIL), vulvar acanthosis with altered differentiation (VAAD), warty LS [50, 55, 56]. In 2017, J. Watkins et al. noted an association between verrucous (warty) and keratinizing squamous cell carcinoma, HPV-negative and many atypical warty lesions, many of which previously defied classification and did not meet the traditional criteria for vulvar intraepithelial neoplasia or other known prior lesions. They proposed the name DEVIL and identified these lesions based on the following signs: exophytic, with pronounced acanthosis or warty hyperplasia; ab-

sence of histomorphological characteristics of HSIL; absence of basal atypia sufficient to diagnose dVIN [36]. VAAD was described by A.F. Nascimento et al. back in 2004 [57], but taking into account similar histological features, J. Watkins et al. proposed to consider it as a form of DEVIL. They showed that these lesions demonstrate a lack of expression of abnormal p53, as well as a significant increase in the PIK3CA mutation. These data were confirmed later, in 2020, in the study of B. Tessier-Cloutier et al. [38]. Due to the rare occurrence, the malignancy potential of noninvasive warty vulvar lesions independent of HPV and *TP53* mutation is still unclear. However, it is likely that they are the precursors of the third molecular subtype of VSCC [55].

DIAGNOSTIC METHODS

Studies on the correlation between the morphological type of carcinoma and HPV status have shown that HPV-positive tumors were more often of the basaloid or warty type, whereas HPV-independent tumors were usually of the keratinizing type [20, 44]. According to a meta-analysis conducted by M.T. Faber et al., the prevalence of HPV DNA in the three main subtypes of VSCC is: 76.5 % – in warty carcinomas; 84.0 % – in basaloid carcinomas; 13.2 % – in the keratinizing type [58]. However, a large study by N. Rakislova et al., which included a large number of tissue samples (1594 paraffin blocks), showed that 36.5 % of HPV-associated carcinomas were of the usual keratinizing type [59]. In their work, A.S. Cheng et al. tried to classify VSCC as HPV-associated and independent of it, assessing the morphology of the tumor, the epithelial precursors adjacent to it and the age of the patients. Even using this multifaceted approach, they misclassified 17 % of cases regarding HPV status [20]. F. Dong et al. obtained similar data in their study, in which the results of molecular studies differed from the morphological assessment of HPV status in 21 % of cases [60]. These data once again emphasize the impossibility of reliably distinguishing HPV-associated and HPV-independent carcinomas only on the basis of morphological examination.

According to studies, immunohistochemical detection of p16 expression has demonstrated a high correlation with the detection of HPV DNA by PCR in cervical, anal, head and neck cancers and is considered a reliable marker of HPV-induced carcinogenesis [21, 61]. However, studies on the correlation between p16-positivity and HPV status in vulvar squamous cell cancer are controversial. Thus, in a large cohort multicenter study involving 1,709 samples, when testing for HPV DNA, the proportion of positive samples was 25.1 % ($n = 429$), while p16-positivity was observed only in 22.4 % ($n = 377$) [62]. J.J. Sznurkowsky et al. in their study showed that among 35 tissue samples with overexpression of p16, HPV DNA was absent in 10 cases (28.6 %), while among 50 tumors without overexpression of p16, HPV DNA was detected in 12 (24.0 %) cases [63]. The absence of DNA virus may be false negative, however, a highly sensitive PCR method with a wide range of primers (more than 68 types) was used in this study. The results obtained can probably be explained by the fact that the inactivation of Rb protein by E7, leading to the expression of p16, is only one potential form of the possible. Data were obtained showing a direct increase in p16 expression with chronological age. During the average life span, this protein increases approximately by 16 times [64, 65]. Thus, since VSCC is diagnosed more often in the age group of 65–70 years [3, 66], the expression of p16 may be due to the ageing rather than to the virus. The authors suggest not to consider only p16-positivity as a surrogate marker of HPV-positive status of VSCC [63].

The study by L. Woelber et al., representing part of a large retrospective study in Germany, showed that out of 411 tumor tissue samples, 204 (49.6 %) were HPV-positive DNA, while p16 expression was detected in 166 (30.2 %) samples. PCR was positive in 85.6 % of p16+ tumors (113 out of 132) and 32.3 % of all p16– tumors. Due to the unclear mutagenesis, cases of p16 discrepancy and HPV DNA detection were excluded from the analysis [24].

However, most authors have demonstrated a convincing correlation between a positive result for HPV DNA in samples and p16 expression with the method with 100 % sensitivity and 98 % specificity [20, 28, 67, 68]. In the study of G. Allo et al., 91 % of PCR-positive cases were also p16-positive; thus, sensitivity and specificity were 91 % and 84 %, respectively [69].

In some studies, the authors used the result of p16 expression as the only marker of the HPV status of VSCC [16, 41, 53]. Due to the fact that overexpression of p16 can occur individually and in different biological contexts, it may be necessary to assess the presence of both markers when determining the functional significance of HPV in carcinogenesis [21, 70].

Since *TP53* is the most frequently mutated gene in HPV-independent carcinogenesis, in 2020 B. Tessier-Cloutier et al. and K.E. Kortekaas et al. published studies showing a high correlation between the interpretation of p53 expression by the IHC method and its mutational status [71, 72]. According to their results, the IHC assessment of p53 expression in vulvar carcinoma samples differs from the interpretation of that in ovarian and endometrial carcinomas [73, 74]. Prior to their study, most authors in their works determined abnormal staining of p53, taking into account only the percentage of stained cells, and did not take into account their distribution among the epithelial layers [7, 60, 75–77]. The new study was able to demonstrate six different staining patterns: four displayed p53mut expression, and two displayed normal (wild) p53wt expression. The authors note that in cases of difficulty in assessing patterns, especially wild-type, interpretation of p53 staining in combination with p16 is necessary.

Thus, using the IHC analysis, three molecular subtypes of VSCC can be distinguished: 1) p16+/p53wt is associated with HPV infection; 2) p16-/p53mut, less frequently p16+/p53mut, corresponds to HPV-independent carcinoma with *TP53* mutation; 3) p16–/p53wt is HPV-independent and it is not associated with the *TP53* mutation. It should be noted that the IHC analysis is also useful for the differen-

tial diagnosis of VIN, since HSIL usually demonstrates block-positive staining of p16 and the expression pattern of p53wt, while dVIN does not show expression for p16 and demonstrates the pattern of mutant p53 [49, 50].

Oncoproteins p16 and p53 are the most studied biomarkers for VSCC diagnosis. The question of the expediency of determining the expression of PDL-1 in immune and tumor cells in vulvar cancer remains less studied. Studies have shown that the expression of this marker does not depend on the HPV status of the tumor. In addition, there is a discrepancy in the definition of expression in the primary and metastatic lesion of carcinoma. [23, 78]. The prognostic value of PD-L1 has not been sufficiently studied. However, the data obtained indicate that its positive expression is largely associated with metastasis of the process to the lymph nodes, as well as with the worst prognosis of overall survival among patients [23, 79].

The prognostic value of p16 and p53. Recent studies show that IHC-determination of p16 and p53 expression can also be used as molecular markers of disease prognosis. In his paper K.E. Kortekaas et al. have shown that the survival rates among the three molecular subtypes of squamous cell carcinomas are different [5]. The overall 5-year survival rate was 83 % (69,9–90,3 %), 64 % (48,9–75,9 %) and 48 % (41.5–55.0 %) for p16+/p53wt, p16–/p53wt and p16–/p53mut VSCC, respectively. A similar pattern was observed for relative survival. Among 275 p16–/p53mut cases there were 119 (43 %) recurrence compared to 16 of 63 (25 %) and 11 of 75 (15 %) for p16–/p53wt and p16+/p53wt, respectively ($p < 0.0001$). However, the time before the occurrence of recurrence did not differ between subtypes. L.S. Nooji et al. also demonstrated better overall and recurrence-free survival for patients with HPV-associated cancer, but the difference was not statistically significant [25]. The five-year survival rate was 75 %, 62.7 % and 56.3 % for patients with tumors p16+/p53wt, p16–/p53wt and p16-/p53mut, respectively ($p = 0.296$). The probability of recurrence was higher in the p16–/p53mut (22.6 %) and p16–/p53wt (16.3 %) groups compared to p16+/p53wt (5.3 %). In a recently published study, L. Woelber and colleagues also studied the clinical significance of p16 and p53 expression [24]. The two-year overall survival rates were 70.4 % (p16–/p53mut), 75.4 % (p16–/p53wt) and 82.5 % (p16+/p53wt) ($p = 0.005$). For two-year recurrence-free survival, the following indicators were obtained: 47.1 % (p16-/p53mut), 60.2 % (p16-/p53wt) and 63.9 % (p16+/p53wt) ($p < 0.001$). The risk of recurrence was 35.0 %, 32.0 % and 22.7 % for the p16–/p53mut, p16–/p53wt and 16+/p53wt groups, respectively. However, given the small number of articles evaluating the outcomes of all three molecular subtypes, further research in this direction is required.

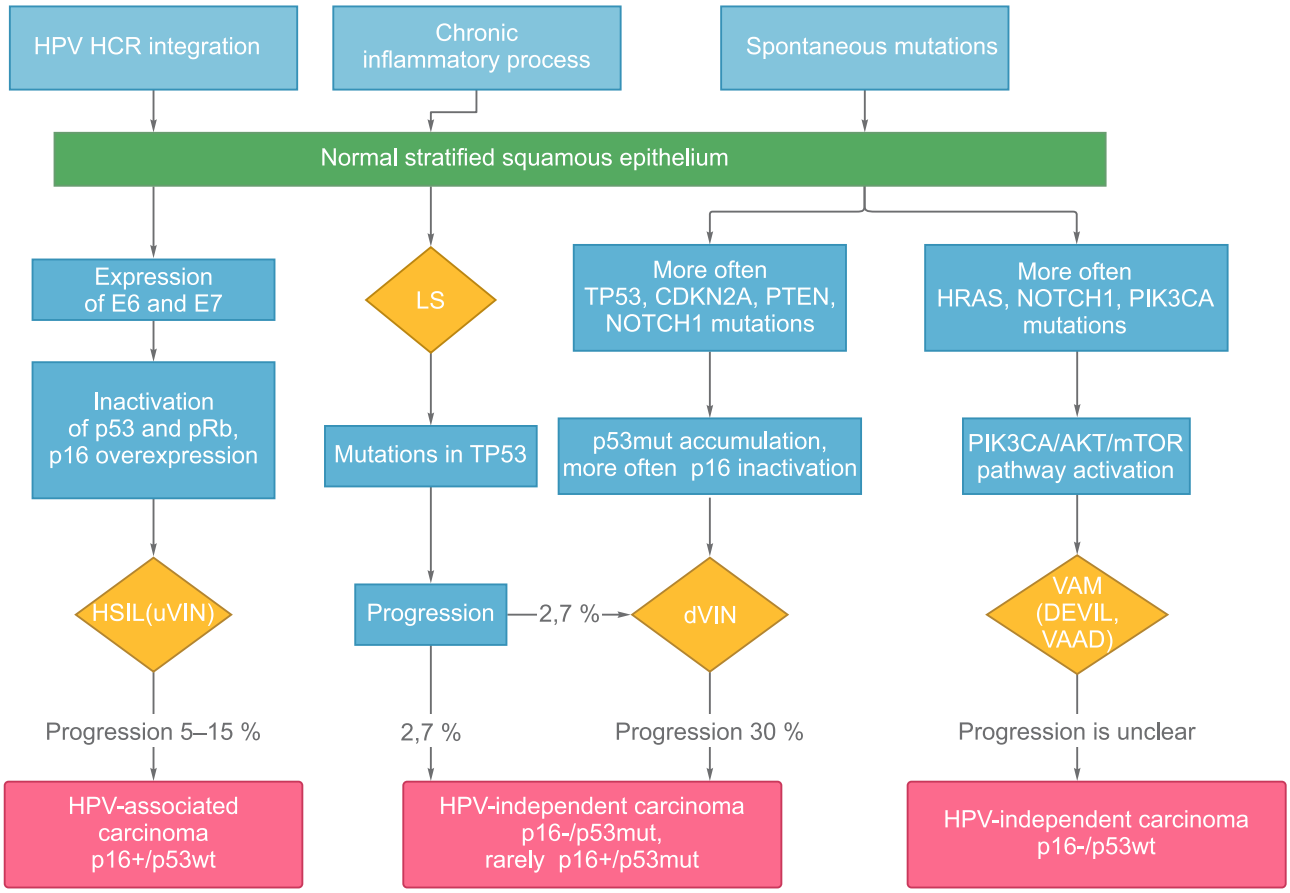

FIG. 1.

Precancerous lesions (HSIL, dVIN and VAM) and molecular subtypes of vulvar squamous cell carcinoma (HPV-associated and HPV-independent) diagnosed by immunohistological analysis of p16 and p53 expression

CONCLUSION

Vulvar squamous cell carcinoma can arise from previous lesions in several pathogenetic pathways, being HPV-associated or HPV-independent. Summarizing the literature data, we have drawn up a diagram of probable pathways of pathogenesis of VSCC (Fig. 1). While morphologically these carcinomas are often similar, the mechanisms of carcinogenesis and precursors, as well as clinical outcomes, are different. Immunohistochemical analysis is a common and affordable method that allows to determine the molecular subtype of carcinoma, as well as to improve the diagnosis of precancerous conditions of VSCC. At the same time, the use of p16 and p53 biomarkers as additional predictors of the outcome of the disease is of great interest. The question of finding new diagnostic and prognostic biomarkers of the disease, for example, PD-L1, remains open. The study of its role in the carcinogenesis of external genitalia deserves attention, since it provides the possibility of using new therapeutic approaches, such as inhibition of immune checkpoints. The possibility of targeting the PI3K/Akt/mTOR pathway with inhibitors should also be considered, whose mutations are common in both HPV-associated and HPV-independent carcinomas. However, given the rare incidence of this disease, further prospective multicenter studies are needed.

Conflict of interest

The authors of this article declare the absence of a conflict of interest.

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