Expression and significance of cyclin D1, cyclin-dependent kinase 4 and cyclin-dependent kinase inhibitor P27 in patients with non-neoplastic epithelial disorders of the vulva

HUAMEI LIU[1], FAN ZHANG[1] and ZONGHENG LI[2]

[1]Department of Gynecology and Obstetrics, Xiangyang Hospital of Integrated Chinese and Western Medicine, Xiangyang, Hubei 441000; [2]Department of Gynecology and Obstetrics, The Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan 646000, P.R. China

Received August 19, 2022; Accepted March 23, 2023

DOI: 10.3892/etm.2023.12055

Abstract. Non-neoplastic epithelial disorders of the vulva (NNEDV) are prevalent and refractory gynecological diseases. However, the underlying pathogenesis of these diseases remain unclear. The present study aimed to investigate the expression and significance of cyclin D1, cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase inhibitor P27 (P27) in patients with NNEDV and provide a reference for clinical diagnosis and treatment. Normal vulvar skin samples from patients with perineum repair (control group, n=20) and skin samples from the vulvar lesions of patients with NNEDV (NNEDV group, n=36) were collected. Expression levels of cyclin D1, CDK4 and P27 were assessed in the samples using immunohistochemistry. The expression of each protein was evaluated based on the mean optical density (MOD). The MODs of cyclin D1 and CDK4 were significantly higher in samples of the three pathological types of NNEDV, namely squamous hyperplasia (SH), lichen sclerosus (LS) and mixed SH and LS lesions, compared with those of the control group. The MOD of P27 was lower in samples of the three pathological types of NNEDV than in the control group, although the difference was not statistically significant. No significant differences in the MOD of cyclin D1, CDK4 and P27 were detected among the three pathological types of NNEDV. The ratios of the MOD of cyclin D1 and CDK4 in the prickle cell layer to those in the basal cell layer were significantly higher in the NNEDV group than in the control group. However, the ratio of the MOD of P27 in the prickle cell layer to that in the basal cell layer exhibited no significant difference between the NNEDV and control groups. NNEDV has the potential for malignant transformation. The occurrence and development of NNEDV may be associated with the acceleration of cell proliferation, in which cyclin D1, CDK4 and P27 contribute to regulation of the cell cycle. Therefore, cyclin D1, CDK4 and P27 may be potential targets in the development of new clinical therapeutic drugs for patients with NNEDV.

Correspondence to: Dr Huamei Liu, Department of Gynecology and Obstetrics, Xiangyang Hospital of Integrated Chinese and Western Medicine, 16 Dongfeng Automobile Avenue, High Tech Zone, Xiangyang, Hubei 441000, P.R. China
E-mail: hmliuswmu@163.com

Abbreviations: NNEDV, non-neoplastic epithelial disorders of the vulva; SH, squamous hyperplasia; LS, lichen sclerosus; HPV, human papillomavirus; CDK, cyclin-dependent kinases; CKI, CDK inhibitor; P27, CKI P27; ISSVD, International Society for the Study of Vulvar Diseases; ISGYP, International Society of Gynecological Pathologists; S-P, streptavidin peroxidase; MOD, mean optical density; VIN, vulvar intraepithelial neoplasia; SCCV, vulvar squamous cell carcinoma

Key words: non-neoplastic epithelial disorders of the vulva, cyclin D1, CDK4, CKI P27, cell cycle

Introduction

NNEDV are a group of chronic diseases characterized by degeneration and pigmentation of the skin and mucosal tissues of the vulva, including squamous hyperplasia (SH), lichen sclerosus (LS) and mixed lesions of SH and LS, which are common refractory gynecological diseases that often cause severe and torturous vulvar pruritus. At present, the pathogenesis of these disorders is unclear, and NNEDV may undergo malignant transformation. As early as 2008, NNEDV were identified as precancerous lesions (1), and it has been reported that vulvar squamous cell carcinomas develop via two different pathways. One is closely associated with human papillomavirus (HPV) infection, which is known as the classical pathway, and the other is associated with inflammatory dermatitis, which is known as the differentiated or simple pathway, in which HPV infection is not involved. The latter accounts for <5% of identifiable vulvar squamous cell carcinomas (1). Inflammatory dermatosis occurs in the epithelium adjacent to the cancerous tissue, which is often continuous with LS or chronic simple lichen disease. NNEDV can become malignant through the differentiated pathway; however, the mechanism by which it becomes malignant remains unknown. Previous studies have explored infection, genetics, hormone levels, autoimmune and metabolic factors, as well as local stimulation, but the

mechanisms of carcinogenesis have yet to be clearly identified (2-4). Molecular biology provides a novel approach to the investigation of the pathogenesis of NNEDV with regard to changes in the mechanism of cell cycle regulation.

The abnormal proliferation of cells caused by dysregulation of the cell cycle is the basis of tumorigenesis and development. The most important cell cycle regulation system of eukaryotic cells is that comprising cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (CKIs). In the normal cell cycle, a cyclin combines with a CDK to form a complex that regulates cell proliferation and differentiation from the G_1 phase to the S phase. However, CKIs inhibit the activity of the cyclin-CDK complexes and block the cell cycle at the G_1 phase, which keeps the system in dynamic equilibrium and maintains the normal operation of the cell cycle. Disruption of the balance of this system can lead to a shortened cell cycle, with uncontrolled cell growth and proliferation, and may also result in tumorigenesis (5).

Cyclin D1, CDK4 and CKI P27 (P27) have been found to be important for the regulation of the cell cycle (5). Therefore, the purpose of the present study was to investigate the expression and significance of cyclin D1, CDK4 and P27 in NNEDV and serve as a reference for the clinical diagnosis and treatment of these disorders.

Materials and methods

NNEDV and control groups. Patients admitted to the Department of Gynecology and Obstetrics of The Affiliated Hospital of Southwest Medical University (Luzhou, China) from August 2020 to July 2021 were enrolled in the present study. A diagnosis of NNEDV was confirmed based on pathology results by two independent pathologists. The inclusion criteria were patients who fulfilled the pathological diagnostic criteria set by the International Society for the Study of Vulvar Diseases (ISSVD) and the International Society of Gynecological Pathologists (ISGYP) in 1987 (6), with clinical manifestations including repeated vulvar pruritus and burning sensation, and who volunteered to participate in the study. The exclusion criteria were patients who were pregnant or lactating, as well as those with a history of hypertension, coronary heart disease, diabetes, hyperlipidemia, thyroid disease, recurrent abortion, adverse pregnancy, immune diseases and malignant tumors, all types of acute vaginitis and acute vulvar infection. In addition, those patients who had received hormone or drug therapy within 1 month, had a mental illness, neurological dysfunction or a long history of smoking were also excluded.

In total, 36 patients [age, 22-75 years; mean age, 39±11.53 years; body mass index (BMI) 18-24 kg/m^2; mean BMI, 21.04±1.06 kg/m^2] were included in the NNEDV group, including 20 cases of SH, 10 cases of LS and 6 cases of mixed lesions. The course of the disease ranged from 6 months to 20 years, with an average of 4.67 years. In the control group, normal vulva skin samples from 20 patients undergoing perineal repair were collected (mean age, 41.10±13.17 years; BMI, 18-24 kg/m^2; mean BMI, 21.01±1.14 kg/m^2). No significant differences in general data between the NNEDV and control groups were identified (P>0.05).

Skin tissues (diameter, 0.5 cm; depth, 0.5 cm) were excised under local anesthesia from patients in the control and NNEDV groups. The samples were immediately fixed in 10% neutral formalin and then embedded in paraffin.

Reagents. The immunohistochemical streptavidin peroxidase (S-P) method was used to detect the expression of cyclin D1, CDK4, and P27 in skin lesions. The primary antibodies used include: i) Rabbit anti-human cyclin D1 monoclonal antibody (cat. no. ZA-0101; 0.1 ml; dilution, 1:50); ii) rabbit anti-human CDK4 monoclonal antibody (cat. no. ZA-0614; 0.1 ml; dilution, 1:150); iii) rabbit anti-human P27 monoclonal antibody (cat. no. ZA-0557; 0.1 ml; dilution, 1:20). The S-P immunohistochemical series kit (cat. no. SP-9000) includes: i) Normal goat serum blocking solution; ii) secondary antibody: biotin labeled sheep anti-rabbit IgG working solution (dilution, 1:1); iii) horseradish enzyme labeled chain enzyme ovalbumin working solution (dilution, 1:1). All the aforementioned reagents were purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.

Main instruments. Leica pathological tissue slicer (Leica Microsystems GmbH), Heating and baking machine (Tk-218; Hubei Xiaogan Taiwei Electronic Equipment Co., Ltd.), refrigerator (Bco-220; Changhong Meiling Co., Ltd.), constant temperature incubator (wk891, 37˚C; Ningbo David Medical Device Co., Ltd.), super high-temperature pressure cooker (diameter, 38 cm; Wuhan Suber Pressure Pot Co., Ltd.), Confocal microscope (Ax70; Olympus Corporation) and image processing system (KS400; Zeiss AG) were used for processing the samples as well as visualizing and analyzing the results.

Detection methods All tissues were fixed in 10% neutral formalin at 36-38˚C for 24 h. After dehydration and being rendered translucent, the tissues were waxed, embedded in paraffin and routinely sectioned to a thickness of 4 μm. Four sections from each tissue were collected, one of which was stained with hematoxylin and eosin at 36-38˚C, the entire dyeing process takes 1 h and 30 sec, while the remaining sections were prepared for immunohistochemistry using the S-P method, which process are as follows: i) Continuously slice paraffin specimens at 4 μm and place in oven at 60˚C for 3-5 h; ii) leave paraffin sections at 22˚C for 5 min, then dipped into xylene I and II for 10 min, respectively; iii) immerse in 100, 95, 90, 80 and 70% alcohol solution for 10 min, respectively; iv) rinse three times with double distilled water for 1 min each time; v) antigen repair: Put the tissue section into antigen repair solution (PH 6.0), steam under high pressure for 3 min and cool to room temperature; vi) rinse with 0.01 M PBS solution (pH 7.4) three times for 10 min each time; vii) endogenous peroxidase was blocked by hydrogen peroxide (3%) and incubated at 37˚C for 10 min; viii) rinse with 0.01 M PBS solution (pH 7.4) three times for 5 min each time; ix) add 5% normal goat serum blocking solution (purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.) and place in a wet box in the incubator at 37˚C for 20 min to eliminate non-specific staining; x) pour out the serum and add the primary antibody (rabbit anti-human cyclin D1, CDK4 or P27 monoclonal antibody). Place in incubator at 37˚C for 30 min and transfer to refrigerator at 4˚C overnight; xi) rewarm the

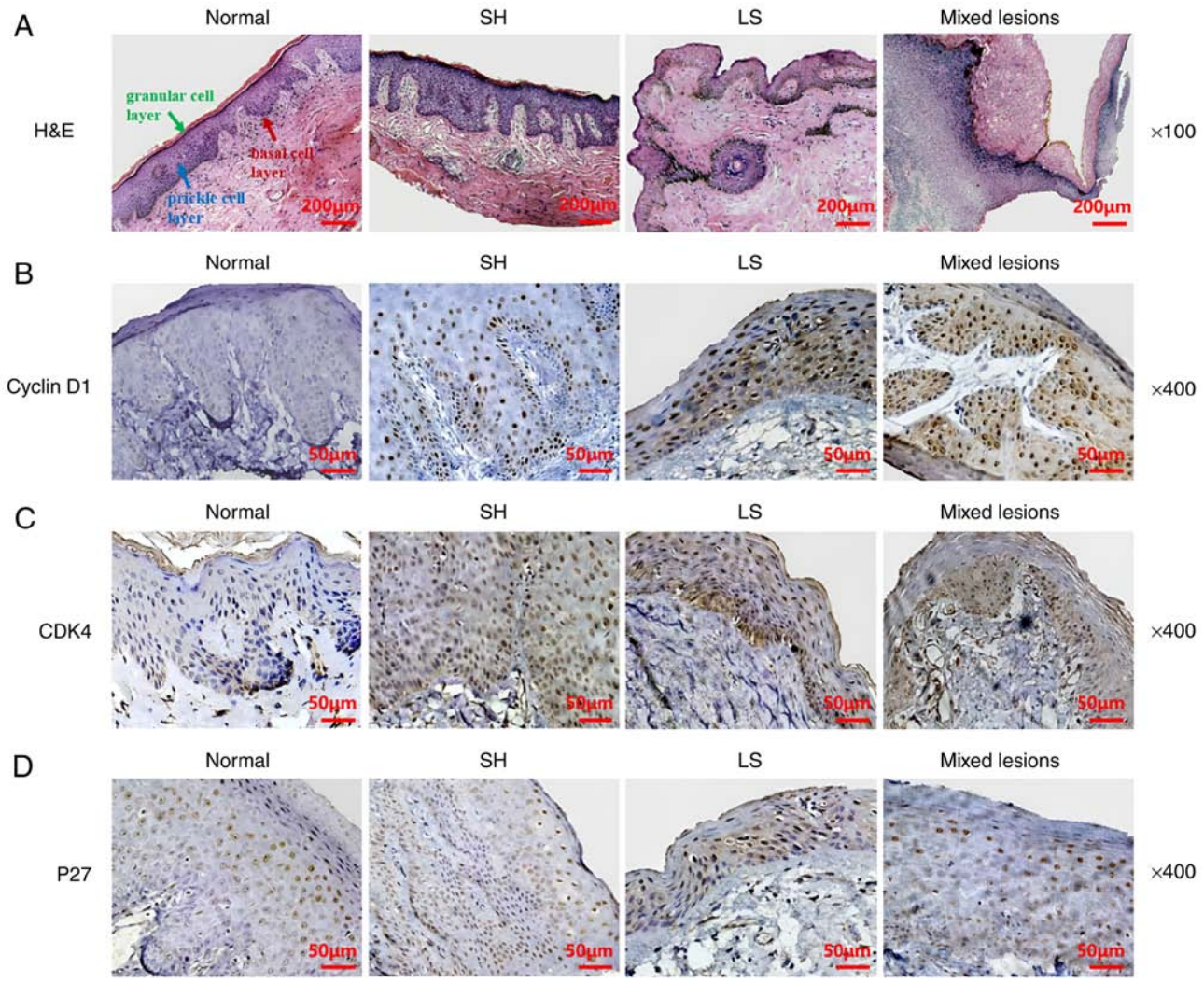

Figure 1. Expression of cyclin D1, CDK4 and P27 in normal vulvar tissue and vulvar tissues from patients with non-neoplastic epithelial disorders of the vulva. (A) H&E staining of normal, SH, LS and mixed lesion tissues, respectively. Scale bars, 200 μm. Immunohistochemical staining of (B) cyclin D1, (C) CDK4 and (D) P27 in normal, SH, LS and mixed lesion tissues, respectively. Scale bars, 50 μm. CDK4, cyclin-dependent kinase 4; P27, cyclin-dependent kinase inhibitor P27; SH, squamous hyperplasia; LS, lichen sclerosus; H&E, hematoxylin and eosin.

following day, rinse with 0.01 M PBS solution three times for 5 min each time; xii) add biotin-labeled secondary antibody (sheep anti-rabbit IgG) working solution dropwise and place in a wet box in the incubator at 37˚C for 25 min; xiii) rinse with 0.01 M PBS solution (pH 7.4) three times for 5 min each time; xiv) add 100 μl of horseradish enzyme labeled chain enzyme ovalbumin working solution and put it in wet box in incubator at 37˚C for 15 min; xv) rinse with 0.01 M PBS solution (pH 7.4) three times for 5 min each time; xvi) DAB color development: Color development at room temperature and control the reaction time under the microscope; xvii) rinse thoroughly with tap water for 10 min; xviii) hematoxylin was counterstained at 25˚C for 5 min, rinse thoroughly with tap water for 5 min, then add hydrochloric acid (0.5%) and alcohol solution to differentiate saturated lithium carbonate returns to blue; xix) use conventional gradient alcohol dehydration, immerse in xylene and seal the sheet with neutral gum. Observe and photograph under the microscope. As the quality control standard, 0.01 M PBS solution (pH 7.4) was used as the negative control instead of the primary antibody.

Pathological diagnostic criteria of NNEDV. Diagnostic criteria jointly created by the ISSVD and ISGYP in 1987 were applied to diagnose NNEDV. The 20 cases of SH exhibited cell proliferation of the squamous epithelium, obvious keratosis and incomplete keratosis of the epidermis, hypertrophy of the prickle cell layer, extended epithelial feet, evident dermal papilla between the epithelial feet, as well as a small amount of lymphocyte and plasma cell infiltration around the blood vessels in the superficial dermis. The 10 cases of LS presented with epidermal hyperkeratosis and keratin embolism of the hair follicles, thinning of the prickle cell layer with liquefaction degeneration of the basal cells, reduced melanocyte counts, the thickening or disappearance of epithelial feet, and homogenization of the dermis, as well as the infiltration of lymphocytes and plasma cells in homogeneous bands. The 6 cases of mixed type presented with the pathological features of both types of lesions (Fig. 1).

Determination of immunohistochemical results. Positive staining was indicated by tan or yellow particles. The nucleus and/or cytoplasm were sites of positive staining for cyclin D1

Table I. Mean optical density of cyclin D1, CDK4 and P27.

Group	Number	Cyclin D1	CDK4	P27
SH	20	2.36±1.62[a]	4.03±1.81[a]	2.31±1.35
LS	10	2.25±1.71[a]	3.69±2.21[a]	2.48±1.11
Mixed lesions	6	2.38±0.92[a]	4.17±1.12[b]	2.35±1.35
Control	20	0.53±0.76	1.71±0.52	4.15±2.33
F-value		3.60	3.67	1.83
P-value		0.03	0.02	0.18

Data are presented as the mean ± standard deviation. [a]P<0.05 vs. the control group; [b]P<0.01 vs. the control group. CDK4, cyclin-dependent kinase 4; P27, cyclin-dependent kinase inhibitor P27; SH, squamous hyperplasia; LS, lichen sclerosus.

and CDK4, while the nucleus was the site of positive staining for P27. For each section, five typical fields were observed using a confocal microscope at a magnification of x100, and photographic images were captured at a magnification of x400. The positive expression of protein was evaluated based on the mean optical density (MOD). Image Pro 6.0 Professional Image Analysis Software (Media Cybernetics, Inc.) was used to assess the MOD. MOD analysis was performed blindly by three different people and the mean value was taken.

Statistical methods. The SPSS Statistics 22.0 software package (IBM Corp.) was used to perform the statistical analysis. Data are expressed as the mean ± standard deviation. Multiple sample means were compared using one-way analysis of variance followed by Tukey's post hoc test for pairwise comparisons. Two sample means were compared using the unpaired t-test, with a=0.05 (bilateral) being set as the test level. P<0.05 was considered to indicate a statistically significant result.

Results

Sites of cyclin D1, CDK4 and P27 expression in normal and NNEDV tissues. Staining for cyclin D1 and CDK4 was observed in the nucleus and/or cytoplasm. In normal tissues, the positive cells were mainly distributed in the epithelial basal cell layer, and also present in the prickle cell layer. However, in NNEDV tissues, the positive cells were mainly distributed in the prickle cell layer. Positive cells were also observed in the granular cell layer in some cases, with cyclin D1 positive cells in 7 cases of SH and 4 cases of LS, as well as CDK4 positive cells in 8 cases of SH, 3 cases of LS and 2 cases of mixed lesions. P27 staining was visible in the nucleus and was mainly distributed in the basal cell and prickle cell layers of the normal and NNEDV tissues (Fig. 1).

Expression intensity of cyclin D1, CDK4 and P27 in normal and NNEDV tissues. The MOD of cyclin D1 in the control, SH, LS and mixed groups was 0.53±0.76, 2.36±1.62, 2.25±1.71 and 2.38±0.92, respectively. The MOD of CDK4 in the control, SH, LS and mixed groups was 1.71±0.52, 4.03±1.81, 3.69±2.21 and 4.17±1.12, respectively. The MOD of P27 in the control, SH, LS and mixed groups was 4.15±2.33, 2.31±1.35, 2.48±1.11 and 2.35±1.35, respectively. The MOD values of cyclin D1 and CDK4 in the three pathological types of NNEDV (SH, LS and mixed lesions) were significantly higher compared with those of the control group (all P<0.05). The MOD values of P27 in the three types of NNEDV were lower than that of the control group, but no significant difference was detected (all P>0.05). No significant difference in the MOD values of cyclin D1, CDK4 or P27 were detected among the three pathological types of NNEDV (all P>0.05; Table I).

Changes in the ratios of the MOD of cyclin D1, CDK and P27 in the prickle cell layer to those in the basal cell layer. The distribution of cyclin D1 and CDK4 positive cells in the epithelium of the control group was different from that of the NNEDV group. The ratio of the MOD of cyclin D1 in the prickle cell layer to that in the basal cell layer was 0.43±0.30 in the control group and 2.29±0.77 in the NNEDV group. The ratio of the MOD of CDK4 in the prickle cell layer to that in the basal cell layer was 0.87±0.50 in the control group and 2.41±0.86 in the NNEDV group. For both cyclin D1 and CDK4, the ratio of the MOD in the prickle cell layer to that in the basal cell layer was significantly higher in the NNEDV group compared with the control group (both P<0.01). The ratio of the MOD of P27 in the prickle cell layer to that in the basal cell layer was 1.56±1.17 in the control group and 1.39±0.77 in the NNEDV group; no significant difference in this ratio was found between the two groups (P>0.05; Table II).

Discussion

Each stage of the cell cycle is precisely regulated. Hartwell *et al* (7) proposed the concept of the cell cycle regulatory checkpoint in the 1970s. There are two key regulatory points in the cell cycle: G_1/S and G_2/M (8). Cell cycle progression is primarily driven by CDKs, which bind to cyclins to form a complex and are activated by phosphorylation or dephosphorylation, thereby promoting the expression of genes associated with the cell cycle (9-12).

To date, a number of CDKs, including CDK1, 2, 4, 6, 7 and 9 and cyclins, including cyclins A, B and D-H, have been identified and are known to play an important role in cell cycle regulation: Cyclin D-CDK4/6 complex initiates the cell cycle process, cyclin E-CDK2 complex regulates entry to the S phase, cyclin A-CDK2 complex regulates S-phase DNA replication and cyclin A/B-CDK1 complex triggers mitosis.

Table II. Ratios of the mean optical density of cyclin D1, CDK4 and P27 in the prickle cell layer to those in the basal cell layer.

Group	Number	Cyclin D1	CDK4	P27
Control	20	0.43±0.30	0.87±0.50	1.56±1.17
NNEDV	36	2.29±0.77	2.41±0.86	1.39±0.77
t-value		9.55	4.08	0.44
P-value		<0.00	0.00	0.67

Data are presented as the mean ± standard deviation. CDK4, cyclin-dependent kinase 4; P27, cyclin-dependent kinase inhibitor P27; NNEDV, non-neoplastic epithelial disorders of the vulva.

Each cyclin-CDK complex can trigger the expression of the next cyclin-CDK complex to regulate the cell cycle at all stages (13,14).

The reason for selecting cyclin D1, CDK4 and P27 for examination in the present study was that cyclin D (cyclin D1/D2/D3) and CDK4/6 are the core molecules that drive the initiation of the cell cycle. Once cyclin D1-CDK4 complexation occurs, the cell cycle is triggered. The abnormal expression of cyclin D1 leads to DNA damage and activation of checkpoint kinase 1, resulting in abnormal cell growth (13,14). P27 is a broad-spectrum CKI that inhibits most CDKs (15,16). When P27 binds to a cyclin D-CDK complex, the activity of the CDK is inhibited and retinoblastoma-associated protein phosphorylation is inhibited, leading to cell-cycle arrest in the G phase and the cessation of cell growth (15,16). In normal tissues, the levels of CDK4, cyclin D1 and P27 are in balance. However, the upregulation of CDK4 and cyclin D, as well as the downregulation of P27, can promote cell division and proliferation disorders, and thereby induce numerous types of human tumors (for example, esophageal squamous cell carcinoma, lung cancer, oral squamous cell carcinoma, renal pelvis and ureter cancer, vulvar cancer, etc.) (17-25). Therefore, investigation of the expression of cyclin D1, CDK4 and P27 in patients with NNEDV is potentially of great importance.

The characteristics and significance of cyclin D1, CDK4 and P27 expression in NNEDV tissues were investigated in the present study. It was observed that the MODs of cyclin D1 and CDK4 in the three pathological types of NNEDV were significantly higher than those in the control group. No statistically significant differences in the MODs of cyclin D1 and CDK4 were detected among the three pathological types. The MOD of P27 in the control group was higher than that in the three pathological groups, but the differences were not found to be statistically significant. Furthermore, no statistically significant difference in the MOD of P27 was detected among the three pathological groups. These results indicate that the proliferation of epithelial cells in NNEDV tissues is likely to be more active than that in normal tissues and suggest that high expression of cyclin D1 and CDK4 plays a key role in the transformation of normal tissue to NNEDV.

Previous studies have confirmed that cyclin D1 and CDK4 are protooncogenes and are upregulated in numerous types of tumors (26-29). One study (20) revealed that the expression levels of cyclin D1 tended to increase as vulvar tissue progressed from normal to NNEDV, vulvar intraepithelial neoplasia (VIN) and vulvar squamous cell carcinoma (SCCV), and the expression of cyclin D1 was significantly increased in vulvar carcinoma. These findings indicate that the high expression of cyclin D1 is associated with the occurrence and development of vulvar carcinoma. In the present study, the expression levels of cyclin D1 and CDK4 were significantly increased in NNEDV tissues compared with normal tissues. This suggests that although NNEDV is a benign lesion, the high expression of cyclin D1 and CDK4 in the lesion may cause the normal cell cycle to become dysregulated and cell proliferation to be activated, ultimately resulting in carcinogenesis. Therefore, the high expression levels of cyclin D1 and CDK4 may be important in the formation of NNEDV, and the possibility of malignant transformation of NNEDV should be monitored clinically. In addition, the expression levels of these proteins could be used as molecular markers to distinguish pathological tissues from normal tissues.

As an important tumor suppressor gene, P27 is a widely studied prognostic factor, and the deletion of P27 is an important index for judging the nature and prognosis of numerous diseases. Kagawa *et al* (30) observed that decreased expression of P27 was associated with the progression and poor prognosis of breast, colon and gastric carcinomas. In addition, Yanagi *et al* (24) observed that P27 was expressed in the normal epidermis, and the expression level of P27 in squamous cell carcinoma (SCC) was significantly lower than that in normal epidermis. Yanagi *et al* also reported that the absence of P27 expression was a frequent event in SCC. In the present study, the expression of P27 in NNEDV was not significantly decreased compared with that in the control tissue and did not differ according to the pathological type of NNEDV. This result is consistent with the findings of Zamparelli *et al* (31), which showed that there was no significant difference in P27 protein expression between normal vulva skin, NNEDV and VIN, although a decreasing trend was observed. However, in another study, Zannoni *et al* (32) reported a significant difference in P27 expression between SCCV and precancerous lesions, and a significant reduction of P27 expression in NNEDV tissues compared with the normal control group, with a decreasing trend in P27 expression in the following sequence: Normal skin tissue-NNEDV-VIN-SCCV.

Based on these findings, it is hypothesized that the high expression of cyclin D1 and CDK4 disrupted the balance between cyclin D1-CDK4 complex and P27 levels, resulting in the epithelial cell proliferation in NNEDV being more active than that in normal tissues, indicating that NNEDV has the potential to undergo malignant transformation. The

occurrence and development of NNEDV may be associated with acceleration of the cell cycle, and cyclin D1, CDK4 and P27 are involved in regulation of the cell cycle in NNEDV.

It is possible that preventing the proliferation of abnormal skin cells via control of the cell cycle, achieved by concurrently inhibiting cyclin D1 and CDK4 as well as promoting the activity of P27, may become a useful molecular strategy for the therapy of NNEDV. As P27 is a broad spectrum CKI with an important role in the G_1 and S phases of the cell cycle, the promotion of P27 activity may be beneficial.

The characteristics and significance of the distribution of cyclin D1, CDK4 and P27 in normal and NNEDV tissues were also investigated in the present study. The results demonstrated that for cyclin D1 and CDK4, the ratio of the MOD in the prickle cell layer to that in the basal cell layer was higher in the NNEDV group compared with the control group ($P<0.001$ and $P=0.001$, respectively). However, for P27, the ratio of the MOD in the prickle cell layer to that in the basal cell layer showed no significant difference between the two groups ($P>0.05$). These results suggest that the distributions of cyclin D1- and CDK4-positive cells in the epithelial tissues of the control and NNEDV groups differed. The cyclin D1- and CDK4-positive cells were mainly distributed in the basal cell layer in normal tissues, which was a normal distribution. In the basal layer, which is also known as the germinal layer, cells divide actively to constantly produce new cells that move up to the stratum corneum where they replenish the aged and shed keratinocytes. Thus, basal cells play a role in epidermal repair and germination. However, in NNEDV tissues, cyclin D1 and CDK4 positive cells were mainly distributed in the prickle cell layer, otherwise known as the stratum spinosum, which suggests that cell division and proliferation in the prickle cell layer were more active than those in the basal cell layer. P27 positive cells were mainly distributed in the basal cell and prickle cell layers of the normal and NNEDV tissues, which is consistent with the findings of some previous studies. For example, Pu *et al* (33) found that the expression of proliferating cell nuclear antigen in LS tissues was mainly distributed in the prickle cell and granular cell layers, with lower levels of expression in the basal cell layer. The results indicated that the thinned prickle layer in LS had stronger proliferative capacity than normal skin. In addition, Liu *et al* (34) found that the prickle cell layer of NNEDV tissues had strong growth and differentiation potential. Therefore, cell proliferation appears to be abnormal in NNEDV tissues, with an imbalance of cell proliferation activity between different layers of the epithelium. The results suggest that the cells in the basal cell layer retained normal proliferation activity whereas those in the prickle cell layer were likely to proliferate more actively. Even in the thinned prickle layer in LS, the proliferative ability of the cells remained strong. Therefore, the change in the balance of epithelial cell proliferation between different layers of NNEDV tissue may indicate the possibility of malignant transformation.

The clinical significance of cyclin D1, CDK and P27 expression in NNEDV merits consideration. Although there are differences in the clinical manifestations and pathological changes of SH, LS and mixed lesions, no significant differences in the MOD of cyclin D1, CDK4 and P27 were detected among these three pathological types and changes in cyclin D1, CDK4 and P27 were not significantly associated with the pathology of NNEDV. Therefore, the three pathological types may have a common or similar pathogenesis. As proto-oncogenes, cyclin D1 and CDK4 play an important role in the occurrence, development, biological behavior and prognosis of some malignant tumors. The results of the present study suggest the possibility of malignant transformation occurring in cases of NNEDV. Therefore, it is recommended that patients with NNEDV are subjected to long-term follow-ups. In addition, cyclin D1 and CDK4 could potentially be used as indicators for the follow-up of lesion progression.

The ultimate purpose of the present study on NNEDV was to provide information useful in the clinic and serve as a basis for clinical drug development, diagnosis and treatment, or further experimental research. The study provides a theoretical basis for the development of more effective treatments for retarding or reversing the progress of NNEDV and preventing malignant transformation. We hypothesize that cyclin D1, CDK4 and P27 are potential targets for the development of new clinical therapeutic drugs for the treatment of patients with NNEDV.

However, the study has certain limitations. Although cyclin D1 and CDK4 are protooncogenes associated with NNEDV, the clinical malignant transformation rate of NNEDV is not high, which indicates that other regulatory may influence the cell cycle. The relationships between other regulatory factors in the cell cycle and the exact mechanism of NNEDV require further evaluation.

Acknowledgements

Not applicable.

Funding

This study was supported by the Young Program of National Natural Science Foundation of China (grant no. 71501035).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HL was responsible for experimental design, conceptualization, research implementation, data analysis/interpretation and administrative, technical and material support. FZ contributed to research implementation, data collection and critical review of the knowledge content of the article. ZL performed data collection and statistical analysis. HL and ZL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of Southwest Medical University (ref. no. 2020-61). This study was conducted in accordance with The Declaration of Helsinki. Written informed consent was obtained from all participants.

Patient consent for publication

Informed consent was obtained from all patients regarding the publication of the data and associated images.

Competing interests

The authors declare that they have no competing interests.

References

1. Nieuwenhof H, Avoort I and Hullu J: Review of squamous premalignant vulvar lesions. Crit Rev Oncol Hematol 68: 131-156, 2008.
2. Ruan L, Xie Z, Wang H, Jiang J, Shi H and Xu J: High-intensity focused ultrasound treatment for non-neoplastic epithelial disorders of the vulva. Int J Gynecol Obstet 109: 167-170, 2010.
3. Pu D and Hou M: Etiology and pathogenesis of white lesions of the vulva. J Practical Obstetrics Gynecol 19: 2-3, 2003 (In Chinese).
4. Li GT, Cao JH and Fu YJ: Expression of cyclin D1 and p16 protein in vulvar white lesion. Zhonghua Fu Chan Ke Za Zhi 41: 322-325, 2006 (In Chinese).
5. Bantie L, Tadesse S, Likisa J, Yu M, Noll B, Heinemann G, Lokman NA, Ricciardelli C, Oehler MK, Beck A, *et al*: A first-in-class CDK4 inhibitor demonstrates in vitro, ex-vivo and in vivo efficacy against ovarian cancer. Gynecol Oncol 159: 827-838, 2020.
6. Le J: Gynecology and Obstetrics (7th edition). People's Health Publishing House 231-235: 2008 (In Chinese).
7. Hartwell L H, Culotti J, Pringle JR and Reid BJ: Genetic control of the cell division cycle in yeast: A model to account for the order of cell cycle events is deduced from the phenotypes of yeast mutants. Science 183: 46-51, 1974.
8. Frederick TJ and Wood TL: IGF-I and FGF-2 coordinately enhance cyclin D1 and cyclin E-cdk2 association and activity to promote G1 progression in oligodendrocyte progenitor cells. Mol Cell Neurosci 25: 480-492, 2004.
9. Du Q, Guo X, Wang M, Li Y, Sun X and Li Q: The application and prospect of CDK4/6 inhibitors in malignant solid tumors. J Hematol Oncol 13:41, 2020.
10. Qie S and Diehl JA: Cyclin D degradation by E3 ligases in cancer progression and treatment. Semin Cancer Biol 67: 159-170, 2020.
11. Billard-Sandu C, Tao YG, Sablin MP, Dumitrescu G, Billard D and Deutsch E: CDK4/6 inhibitors in P16/HPV16-negative squamous cell carcinoma of the head and neck. Eur Arch Otorhinolaryngol 277: 1273-1280, 2020.
12. Deng L, Xu M, Fu L, Du G and Wang L: Effects of CDK4/6 inhibitor palbociclib on proliferation, cell cycle and apoptosis of human melanoma cells. Tumor 40: 521-530, 2020 (In Chinese).
13. Liu J, Peng Y and Wei W: Cell cycle on the crossroad of tumorigenesis and cancer therapy. Trends Cell Biol 32: 30-44, 2022.
14. Bury M, Le Calvé B, Ferbeyre G, Blank V and Lessard F: New insights into CDK regulators: Novel opportunities for cancer therapy. Trends Cell Biol 31: 331-344, 2021.
15. Janumyan Y, Cui Q, Yan L, Sansam CG, Valentin M and Yang E: G0 function of BCL2 and BCL-xL requires BAX, BAK, and p27 phosphorylation by mirk, revealing a novel role of BAX and BAK in quiescence regulation. J Biol Chem 283: 34108-34120, 2008.
16. Lee S, Kim S, Chung H, Moon JH, Kang SJ and Park CG: Mesenchymal stem cell-derived exosomes suppress proliferation of T cells by inducing cell cycle arrest through p27kip1/Cdk2 signaling. Immunol Lett 225: 16-22, 2020.
17. Kalu NN, Johnson FM: Do CDK4/6 inhibitors have potential as targeted therapeutics for squamous cell cancers? Expert Opin Investig Drugs 26: 207-217, 2017.
18. Zhou J, Wu Z, Wong G, Pectasides E, Nagaraja A, Stachler M, Zhang H, Chen T, Zhang H, Liu JB, *et al*: CDK4/6 or MAPK blockade enhances efficacy of EGFR inhibition in oesophageal squamous cell carcinoma. Nat Commun 8: 13897, 2017.
19. Fang L, Xu X, Zheng W, Wu L and Wan H: The expression of microRNA-340 and cyclin D1 and its relationship with the clinicopathological characteristics and prognosis of lung cancer. Asian J Surg 44: 1363-1369, 2021
20. Laisom A, Pukhrambam G, Shameen Y, Akoijam N and Sen R: Immunohistochemical study on pten and cyclin D1 in non-neoplastic and neoplastic endometrial lesions. Int J Med Biomed Studies 5: 2021.
21. Thambiah LJ, Bindushree RV, Anjum A, Pugazhendi SK, Babu L and Nair RP: Evaluating the expression of p16 and p27 in oral epithelial dysplasias and oral squamous cell carcinoma: A diagnostic marker for carcinogenesis. J Oral Maxillofac Pathol 22: 59-64, 2018.
22. Kamai T, Takagi K, Asami H, Ito Y, Arai K and Yoshidal KI: Prognostic significance of p27Kip1 and Ki-67 expression in carcinoma of the renal pelvis and ureter. BJU Int 86: 14-19, 2000.
23. Hao S, Wu X and Li H: Expression and significance of cyclin D1 in vulvar carcinogenesis. China Modern Doctor 47: 15-16, 2009 (In Chinese).
24. Yanagi T, Hata H, Mizuno E, Kitamura S, Imafuku K, Nakazato S, Wang L, Nishihara H, Tanaka S and Shimizu H: PCTAIRE1/CDK16/PCTK1 is overexpressed in cutaneous squamous cell carcinoma and regulates p27 stability and cell cycle. J Dermatol Sci 86:149-157,2017.
25. Shi X, Zhu K, Ye Z and Yue J: VCP/p97 targets the nuclear export and degradation of p27Kip1 during G1 to S phase transition. FASEB J 34: 5193-5207, 2020.
26. Li Y, Xiao X, Chen H, Chen Z, Hu K and Yin D: Transcription factor NFYA promotes G1/S cell cycle transition and cell proliferation by transactivating cyclin D1 and CDK4 in clear cell renal cell carcinoma. Am J Cancer Res 10: 2446-2463, 2020.
27. Zhou L, Liu R, Liang X, Zhang S, Bi W, Yang M, He Y Jin J, Li S, Yang X, *et al*: lncRNA RP11-624L4.1 is associated with unfavorable prognosis and promotes proliferation via the CDK4/6-cyclin D1-Rb-E2F1 pathway in NPC. Mol Ther Nucleic Acids 22: 1025-1039, 2020.
28. Nawroth R, Koch J, Mantwill K, Hindupuhr S and Holm PS: CDK4/6 inhibitors improve therapy of oncolytic adenovirus by manipulation of RB-E2F regulated transcription. Urologic Oncol 38: 908-909, 2020.
29. Li W, Li Y, Wang Z, Ren H, Zhang Y, Yang P, Pan S and Wang Y: Genistein promotes the proliferation of ovarian cancer OVCAR-5 cells by upregulating cyclin D1 and CDK4 expressions. J Xian Jiaotong University (Medical Sciences) 42: 59-64, 2021 (In Chinese).
30. Kagawa Y, Yoshida K, Hirai T and Toge T: Significance of the expression of p27Kip1 in esophageal squamous cell carcinomas. Dis Esophagus 13: 179-184, 2000.
31. Zamparelli A, Masciullo V, Bovicelli A, Santini D, Ferrandina G, Minimo C, Terzano P, Costa S, Cinti C, Ceccarelli C, *et al*: Expression of cell-cycle -associated proteins pRB2/p130 and p27kip in vulvar squamous cell carcinomas. Hum Pathol 32: 4-9, 2001.
32. Zannoni GF, Faraglia B, Tarquini E, Camerini A, Vrijens K, Migaldi M, Cittadini A and Sgambato A: Expression of the CDK inhibitor p27kip1 and oxidative DNA damage in non-neoplastic and neoplastic vulvar epithelial lesions. Mod Pathol 19: 504-513, 2006.
33. Pu D, Yin L, Hu F and Li N: DNA content, PCNA and P53 expression vulve lichen sclerosus and their relation with cell proliferation. Chinese J Histochem Cytochem 2: 169-173, 2001 (In Chinese).
34. Liu H, Xue X, Su B and Zhang X: Expression and significance of EGFR a and anti-oncogene protein p53 in vulva dystrophy and carcinoma of old women. China J Modern Med 7: 4-7, 2004 (In Chinese).