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Cyclins D1, D3, E, and A in vulvar carcinoma patients

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Abstract

Objective. The treatment of vulvar squamous cell carcinoma patients is often mutilating. Effort is being made to individualize treatment in order to reduce negative side effects for patients with good prognosis. Molecular markers have been able to predict patient outcome in several tumors. The aim of this study was to characterize the expression of cyclins D1, D3, E, and A in a comparatively large series of patients with vulvar squamous cell carcinoma and look for prognostic impact.

Methods. A total of 224 vulvar squamous cell carcinomas were immunohistochemically investigated for expression of cyclins D1, D3, E, and A using the biotin-streptavidin-peroxidase method and the OptiMax Plus automated cell staining system.

Results. High protein levels of cyclin D1 (any positive nuclei) were found in 58 (26%) cases, cyclin D3 (≥50% positive nuclei) in 61 (27%) cases, cyclin E (≥50% positive nuclei) in 41 (18%) cases, and cyclin A (≥5% positive nuclei) in 156 (70%) cases. No prognostic impact was found for the cyclins D1, D3, E, or A.

Conclusions. The high number of cases showing increased levels of cyclin A suggests that this protein may be important in the pathogenesis of vulvar squamous cell carcinoma. No prognostic impact was found for the cyclins D1, D3, E, or A.

Keywords: Immunohistochemistry; Cyclin A; Cyclin D1; Cyclin D3; Cyclin E; Cell cycle; Squamous cell carcinoma

Introduction

Vulvar carcinoma has an incidence of 1–2 per 100 000 person-years [1]. It has traditionally been considered a disease of elderly women, but in recent years an increasing incidence among younger women has been observed [2,3]. Most patients are treated with surgery, although nonsurgical options including chemotherapy, radiation, and novel topical treatments are available (reviewed in [4]) [5–8]. The treatment in many cases is mutilating with both physical and psychological long-term consequences [1]. Thus, effort is being made to individualize treatment procedures in order to reduce negative side effects for patients with good prognosis [9–11]. The ability to select patients profiting from less radical treatment without increasing the risk of metastasizing and relapse would be helpful in treatment planning. In this sense, molecular markers have been shown able to predict patient outcome in several tumors [12].

The cell cycle is regulated by cyclins, cyclin dependent kinases (cdk's), and cyclin-dependent kinase inhibitors. For the cell to transform from G1- to S-phase, the phosphorylation of pRb in order to release the transcription factor E2F is an essential step, known to be the main function for the cyclin–cdk complexes. To start the cell cycle, mammalian cells require complexes composed of cyclin D and cdk4 or cdk6. The three types of D-cyclins, D1, D2, and D3, are in part cell-type specific, with most cells expressing D3 and either D1 or D2 [13]. Cyclin E forms complexes with cdk2 and phosphorylates pRb in late G1 but is expected to play multiple roles in promoting S-phase. At least one of the critical events controlled by cyclin E is independent of the pRb/E2F pathway and sufficient, when hyperactivated, to bypass the requirement for E2F activity [14]. Cyclin A forms complexes with cdk2 during S phase and with cdc2 in

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G2–M transition. It phosphorylates pRb and the nuclear membrane protein laminin, facilitating nuclear membrane disruption [15].

Previously, overexpressions of cyclins D1, D3, E, and A proteins have been demonstrated in many tumors and correlated with prognosis [16–23]. In previous studies of vulvar lesions and neoplasia, it has been suggested that cyclin D1 is involved in the progression of vulvar cancer, but no prognostic significance was reported [24,25]. To our knowledge, the prognostic impact of cyclins D3, E, and A has not previously been examined in vulvar carcinomas.

To elucidate further the molecular pathogenesis of vulvar carcinomas and to identify molecular markers predictive of patient's outcomes, which would assist clinicians in stratifying women into risk groups, we tested the expression of the cyclins D1, D3, E, and A in a comparatively large series of patients with vulvar squamous cell carcinoma.

Materials and methods

Patient materials

A retrospective study on 224 patients with squamous cell carcinoma of the vulva, undergoing surgery at the Norwegian Radium Hospital in the period 1977–1991, was performed. No chemo- or radiotherapy was given prior to surgery. Postoperative irradiation was given to 46 (21%) of the patients. The median age at diagnosis was 70 years (range 27–96). All patients were followed until death or until 5 years after inclusion. Of the 224 patients, 117 (52%) had 5-year follow-up. The median follow-up was 60 months (range 0.76–60). Sixty-five patients (29%) died of vulvar cancer. Of the 110 patients staged FIGO I and II, eighteen (16%) deaths from vulvar cancer were observed within the first 5 years after diagnosis. Clinical data and follow-up information were obtained from medical records and Statistics Norway. The Norwegian Board of Health and The Data Inspectorate approved the study. Tumors were staged according to the International Federation of Gynecology and Obstetrics (FIGO) classification [26]. Twenty-seven patients did not undergo lymph node dissemination and consequently could not be staged according to the surgical FIGO staging used in the present study. A detailed description of tumor characteristics is given in Table 1.

Histologic specimens were reviewed by one of the authors (J.M.N.) who had no access to clinical information. The tumors were classified and graded according to World Health Organization recommendations [27]. Depth of stromal invasion was measured by an ocular micrometer from the top of the nearest dermal papilla to the deepest point of stromal penetration. Vessel invasion was observed within unquestionably endothelium-lined spaces. As controls, samples of normal vulva were collected from 10 patients undergoing surgery for benign gynecological diseases. In two previous studies, the expression of p53, p16, p21, and p27 has been investigated in the patient material used in the present study [28,29]. The results of these studies have been implemented as pathologic variables in the analyses performed.

Immunohistochemistry

Five-micrometer-sections from formalin-fixed and paraffin-embedded tissues were cut, mounted on glass slides, and dried at 56°C for 30 min and overnight at 37°C. Sections for immunohistochemistry were stained using the biotin-streptavidin-peroxidase method (Supersensitive Immunodetection System, LP000-UL, Biogenex, CA, USA) and OptiMax Plus Automated Cell Staining System (Biogenex). Deparaffinized sections were microwaved in 1 mM EDTA pH 8.0 to unmask the epitopes, and treated with 1% hydrogen peroxide (H_2O_2) for 10 min to block endogenous peroxidase. The sections were incubated with monoclonal antibodies cyclin A (clone 6E6, 1:75, 0.6 μg IgG_1/ml, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK), cyclin D1 (clone DCS-6, 1:200, 0.5 μg IgG_{2a}/ml, Oncogene Research Products, MA, USA), cyclin D3 (clone DCS-22, 1:50, 6.8 μg IgG_1/ml, DAKO, Glostrup, Denmark), and cyclin E (clone 13A3, 1:100, 2.8 μg IgG_{2a}/ml, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) for 30 min at room temperature. The sections were then incubated with a biotin-labeled secondary antibody (1:30) and streptavidin-peroxidase (1:30) for 20 min each. The sections were stained for 5 min with 0.05% 3′3-diaminobenzidine tetrahydrochloride (DAB) freshly prepared in 0.05 M tris (hydroxymethyl)-aminomethane (Tris) buffer at pH 7.6, containing 0.024% H_2O_2 and then counterstained with hematoxylin, dehydrated, and mounted in Diatex. All the dilutions of primary antibodies, biotin-labeled secondary antibody, and streptavidin-peroxidase were made with phosphate-buffered saline (PBS) pH 7.4, containing 1% bovine serum albumin.

All series included positive controls. Negative controls included replacement of the monoclonal antibody with mouse myeloma protein of the same subclass and concentration as the monoclonal antibody. All controls gave satisfactory results. Only distinct nuclear staining was considered positive. Sections were scored by two independent observers (SK and RH) with no knowledge of clinical data. Conflicting results were reviewed until final agreement was achieved. Four semiquantitative classes were used to describe the number of positively stained tumor cells; none, <5% of the cells; 5–50% of the cells; and >50% of the cells. Based upon staining pattern observed in normal vulvar epithelium, cutoffs for each of the proteins were defined. Protein levels were classified as high when any cyclin D1 staining was seen in the tumor, ≥5% of cells were positive for cyclin A, and >50% of cells were positive for cyclin D3 and cyclin E. The κ-values for intra- and inter-observer variability were determined on a subset of 35 patients and were 0.76 and 0.78, respectively, when four semiquantita-