Croat Med J 2005;46(3):436-442

Invasive Squamous Cell Carcinoma of Vulva: Prognostic Significance of Clinicopathologic Parameters

Marin Nola, Anto Blažanović[1], Snježana Dotlić, Anamarija Morović, Ivana Tomičić, Stanko Jukić

Department of Pathology, Zagreb University Hospital Center, Zagreb; and [1]Department of Pathology, General Hospital Vukovar, Vukovar, Croatia

Aim To investigate the prognostic significance of several clinicopathologic parameters in patients with invasive squamous cell carcinoma of the vulva.

Methods We retrospectively studied 43 patients with invasive squamous cell carcinoma of the vulva treated with radical vulvectomy at the Department of Gynecology and Obstetrics at Zagreb University School of Medicine, Croatia, in the period from 1978-1996. At the time of analysis, follow-up information was obtained for all patients, 18 (41.9%) of whom have died and 25 (58.1%) who were alive at the time of the last contact. The mean follow-up time of surviving patients was 121 months (range, 6-216 months). Cox proportional hazard analysis was performed to examine the prognostic significance of age, menstrual status, clinical stage, diameter and localization of the tumor, histological grade, nuclear grade, depth of tumor invasion, presence of vascular space invasion, tumor growth pattern, presence of lymph node metastasis, DNA ploidy, proliferative activity, and mode of therapy.

Results The overall 5-year survival for this group of patients was 62.3%. The results of univariate statistical analysis confirmed that statistically significant prognostic parameters included the age of patients ($P=0.038$), clinical stage ($P=0.001$), nuclear grade ($P=0.002$), the depth of tumor invasion ($P<0.001$), and presence of lymph node metastasis ($P=0.001$). On the other hand, the results of multivariate statistical analysis showed that only the depth of tumor invasion ($P<0.001$) can be considered independent, statistically significant prognostic parameter.

Conclusion Our data suggest that the depth of tumor invasion represents the most important prognostic parameter in the group of patients with invasive squamous vulvar carcinoma. Clinical significance of DNA ploidy and proliferative activity was not found.

Invasive squamous cell carcinoma accounts for approximately 90% of all primary vulvar neoplasms (1-2). Although the anatomic localization of this tumor allows early diagnosis and considerably easy surgical accessibility, the prognosis for these patients remains relatively grim. The explanation for this finding should be sought in the fact that most of the patients are diagnosed in the advanced clinical stage of the disease. Typically, when it occurs in older women, in their seventh or eighth decade of life, it is unrelated to human papillomavirus (HPV). Younger patients appear to have a significantly better prognosis, and the disease is associated with HPV (1,2). Although radical vulvectomy, a complex and debilitating procedure, was previously believed to be the only effective treatment for vulvar cancer (1,2), recent studies have been focused on determining reliable prognostic parameters that would allow individualized, and hopefully simpler, therapeutic modalities to be applied in a certain group of patients with the same effectiveness (e.g. more conservative operative procedures for small, unilateral tumors). According to some authors (3-5), modified

radical procedures are equally effective as the *en bloc* radical vulvectomy for the management of early vulvar cancer, and consequently, scientists have been trying to discover the most important prognostic parameters for survival of these patients. Different reports investigated the influence of various parameters on patient survival such as clinical stage of disease (4-13), older age (6,13), smoking (1,2,13), location and focality (5,13,14), diameter of the tumor (5-7,13,14), histological grade (7,10,14), nuclear grade (6,11,15,16), depth of tumor invasion (6,11,14), presence of vascular space invasion (6,7,14), pattern of tumor growth (7,11,14), and presence of lymph node metastasis (5,6,11,13,14,17). DNA flow cytometric analysis measuring DNA ploidy and proliferative activity have proven to be of prognostic significance for overall patient survival in different gynecological cancers (18-21). However, these methods have not been applied extensively in the study of patients with invasive carcinoma of the vulva (8-11).

The aim of this study was to determine the most important clinical and histopathological parameters which influence the prognosis and outcome of patients with invasive squamous cell carcinoma of the vulva in order to offer them the most suitable mode of therapy. Moreover, it was designed to assess clinical significance of DNA content analysis by flow cytometry.

Patients and Methods

The medical records of patients with invasive squamous cell vulvar cancer who were treated with radical vulvectomy including bilateral groin dissection from 1978 to 1996 at the Department of Gynecology and Obstetrics at the Zagreb University School of Medicine were reviewed. To assess the influence of various prognostic factors on survival, the study included clinical and pathohistological parameters, as well as the results of flow cytometric DNA analysis of paraffin-embedded tumor samples from all cases.

Clinicopathological parameters focused on the age of patients, menstrual status, clinical stage of the disease, diameter and localization of the tumor, histological grade, nuclear grade, depth of tumor invasion, presence of vascular space invasion, tumor growth pattern, presence of lymph node metastasis, and mode of therapy, whereas flow cytometric DNA analysis comprised DNA ploidy and proliferative activity.

Clinical Data

The age of patients, menstrual status, diameter and localization of the tumor, and mode of therapy were determined by medical record review alone. Advanced age, postmenopause, diameter of tumor greater than 20 mm, and clitoral involvement were considered prognostically unfavorable. Clinical staging was done according to the guidelines of the International Federation of Gynecologists and Obstetricians (FIGO) (1,2). Stages I and II were considered favorable and stages III and IV unfavorable. All patients were treated with radical vulvectomy and almost half of them received adjuvant radiotherapy. At the time of the analysis, follow-up information was obtained for all patients, 18 (41.9%) who have died and 25 (58.1%) who were alive at the time of the last contact. The mean follow-up time of surviving patients was 121 months (range, 6-216 months).

Histopathology

A revision of all histological data was performed by two pathologists (M.N. and A.B.), based on all hematoxylin and eosin-stained slides, without the knowledge of the clinical outcome. The following histopathological features were assessed: histological grade, nuclear grade, depth of tumor invasion, presence of vascular space invasion, tumor growth pattern, and presence of lymph node metastasis.

Histological and nuclear grades were determined separately. Histological grading was done according to the guidelines of the Gynecologic Oncology Group (GOG) (1). GOG advocates a system according to the percentage of undifferentiated cells. Grade 1 tumors (H1) have no undifferentiated cells, grade 2 tumors (H2) contain less than 50% undifferentiated cells, grade 3 tumors (H3) have more than 50% but less than 100%, and grade 4 tumors (H4) are almost entirely composed of undifferentiated cells. Only histological grade 1 was designated a favorable prognostic parameter.

Nuclear grade was based on the aspect of the nuclei and was evaluated in the most atypical area. Cells with relatively uniform sized oval nuclei, abundant cytoplasm, minimal nuclear pleomorphism, without prominent nucleoli and evenly dispersed chromatin were considered nuclear grade 1 (N1). Cells with markedly enlarged nuclei displaying irregular coarse chromatin and

prominent eosinophilic nucleoli, with minimal amount of cytoplasm, and marked nuclear pleomorphism were considered nuclear grade 3 (N3), whereas those displaying features between grades N1 and N3 were designated nuclear grade 2 (N2) (11,16). Nuclear grades 1 (N1) and 2 (N2) were designated favorable, and nuclear grade 3 (N3) unfavorable prognostic parameter.

The depth of tumor invasion was measured with an ocular micrometer from the epidermo-stromal junction of the most superficial dermal papilla adjacent to the tumor to the deepest point of tumor invasion. At times, this was not possible because of either a large confluent lesion or wide ulcers. In these cases, the depth was assessed by subtracting the thickness of the adjoining epithelium from the total depth of the carcinoma. The depth of stromal invasion of ≤5 mm was designated favorable prognostic parameter.

The presence or absence of vascular space involvement by the tumor and lymph node metastasis was evaluated on hematoxylin and eosin-stained slides.

The pattern of tumor invasion was separated into confluent pattern and spray pattern. Confluent pattern was defined by a smoothly invading front, whereas spray pattern was characterized with multiple tongues of tumor penetrating the underlying stroma.

Flow Cytometry

For DNA analysis, paraffin blocks which consisted of at least 30% tumor tissue were selected, as documented with the corresponding hematoxylin-eosin slide. Three 40-µm thick sections were cut from the paraffin block. The sections were placed in xylene and then rehydrated in decreasing ethanol concentrations (100%, 95%, and 70%), followed with a final wash in distilled water (22). Tissue was then suspended in a suspension of 0.5% pepsin (Sigma, St. Louis, MO, USA) in 0.9% sodium chloride (pH 1.5) and incubated in a shaking water bath at 37 °C for 1 h. Cells were washed in RPMI medium, filtered through 42-µm nylon mesh and centrifuged 5 min at 800 G. The extracted nuclei were stained for DNA content after a modification of method described by Vindelov et al (23). Ribonuclease S (Sigma), in the final concentration of 1 mg/mL, was added to approximately 1×10^6 nuclei and incubated in a water bath at 37 °C for 30 min. Afterwards, nuclei were resuspended in 50 µg/mL propidium iodide (Sigma, St. Louis, MO, USA) and incubated for 30 min, in the dark, at room temperature. Control tissues comprised normal vulvar tissue, which was always included in the sample, as an inner control. Cellular DNA content and proliferative activity were analyzed on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA), using 15-milliwatt argon ion laser, producing excitation wavelength of 488 nm. For each DNA content analysis, 20,000 nuclei were counted. DNA histograms were analyzed with ModFit LT program (Verity Software House, Inc., Topsham, ME, USA).

A tumor was classified as DNA diploid if only one GO/G1 peak was observed in the expected region of the histogram. If more than one GO/G1 peaks were seen, the tumor was considered DNA aneuploid (ie, DNA index was not used for the definition of DNA aneuploidy). DNA tetraploid tumors were included in the DNA aneuploid group. Those tumors were separated from DNA diploid tumors because the percentage of cells in the G2/M fraction was higher than 15%, and their DNA index was 1.9-2.1. Proliferative activity was defined as the sum of the cell percentages in the S- and G2/M phases. Tumors with proliferative activity higher than 10% were shown to have worse prognosis according to the statistical analysis (data not shown). The coefficient of variation of the G0/G1 peak was always less than 8%.

Exclusion Criteria

To be eligible for the study, a patient had to be initially operated in our hospital for a previously untreated squamous cell carcinoma of the vulva. Patients with incomplete clinical documentation were excluded from this analysis. Superficially invasive squamous cell carcinomas with stromal invasion 1 mm or less and tumor diameter 2 cm or less were not included in the study. The patients for whom appropriate flow cytometric histograms could not be obtained were also excluded from the study. At the end, only 43 patients fulfilled all of the requirements and entered the study.

Statistical Analysis

Survival time was measured in months from the date of diagnosis to the date of death or the last follow-up. Only the deaths caused by the tumor were considered as deaths in the survival analysis, and all others were considered censored.

Actuarial survival probability curves were constructed using the Kaplan-Meier method and compared using log-rank nonparametric test. Categorical (nominal) parameters were analyzed as dummy variables in the case of a binary split, or were coded as ordinal (1, 2, etc.) when appropriate. The Cox proportional hazards regression model with forward stepwise variable selection was used to assess the relative effect of age, menstrual status, clinical stage, diameter and localization of the tumor, histological grade, nuclear grade, depth of tumor invasion, tumor growth pattern, presence of lymph node metastasis, DNA ploidy, proliferative activity, and mode of therapy on survival prognosis. In all statistical analyses, only probabilities lower than 5% ($P<0.05$) were considered significant. Statistical analyses were performed using MedCalc (MedCalc ver. 7, Frank Schoonjans, Mariakerke, Belgium) and SPSS (SPSS for Windows ver. 7, SPSS Inc., Chicago, IL, USA) software.

Results

Clinical and Pathological Findings

The median age of patients with invasive squamous cell vulvar carcinoma was 63 years (range 22-78). With regard to the menstrual status, they were divided into two groups: the menstruating group included 6 patients and the postmenopausal group consisted of 37 patients. According to the clinical stage, there were 22 patients in the favorable and 21 patients in the unfavorable group (Table 1). Clitoris was involved in 11 patients. Almost 80% of patients had tumor larger than 20 mm. Twenty-two patients had well-differentiated (H1) and 21 had moderately (H2) or poorly-differentiated tumors (H3). None of the patients had a tumor designated as grade 4 (H4), ie entirely composed of undifferentiated cells. There were 19 patients with tumors composed of cells with nuclear grade 1 or 2 (favorable group) and 24 patients with nuclear grade 3 (unfavorable group). There were 28 patients with the depth of stromal invasion 5 mm or less, whereas 15 patients had tumors with deeper invasion. Vascular space invasion was visualized in only 3 cases. Confluent growth pattern was obvious in 34 patients. Half of the patients had metastasis in their lymph nodes.

There were 30 patients with tumors in the DNA diploid and 13 patients in the DNA aneuploid group. Thirty-four patients had tumors with low proliferative activity and 9 patients had tumors with high proliferative activity.

Table 1. Clinicopathologic characteristics and cell cycle parameters in 43 patients with invasive squamous cell vulvar carcinoma

Parameters	No. (%) of patients
Menstrual status:	
menstruating	6 (14.0)
postmenopause	37 (86.0)
Clinical stage:*	
I+II	22 (51.2)
III+IV	21 (48.8)
Tumor diameter:	
≤20 mm	9 (20.9)
>20 mm	34 (79.1)
Clitoral involvement:	
no	32 (74.4)
yes	11 (25.6)
Histological grade:†	
I	22 (51.2)
II+III	21 (48.8)
Nuclear grade:‡	
I+II	19 (44.2)
III	24 (55.8)
Depth of invasion:	
≤5 mm	28 (65.1)
>5 mm	15 (34.9)
Vascular space invasion:	
no	40 (93.0)
yes	3 (7.0)
Tumor growth pattern:	
confluent	34 (79.1)
spray	9 (20.9)
Lymph node metastasis:	
no	22 (51.2)
yes	21 (48.8)
DNA ploidy:	
diploidy	30 (69.8)
aneuploidy/tetraploidy	13 (30.2)
Proliferative activity:§	
S+G2/M ≤10%	34 (79.1)
S+G2/M >10%	9 (20.9)
Therapy:	
surgery+linac	21 (48.8)
surgery	22 (51.2)

*Clinical staging was done according to the guidelines of the International Federation of Gynecologists and Obstetricians (FIGO) (1,2).
†Histological grading was done according to the guidelines of the Gynecologic Oncology Group (GOG) (1).
‡Cells with relatively uniform sized oval nuclei, abundant cytoplasm, minimal nuclear pleomorphism, without prominent nucleoli and evenly dispersed chromatin were considered nuclear grade I. Cells with markedly enlarged nuclei displaying irregular coarse chromatin and prominent eosinophilic nucleoli, with minimal amount of cytoplasm, and marked nuclear pleomorphism were considered nuclear grade III whereas those displaying features between grades I and III were designated nuclear grade II (11,16).
§Proliferative activity was defined as the sum of the cell percentages in the S- and G2/M phases (18).

Survival Analysis

Five-year survival of our selected population of patients was 62.3±7.8% (Fig. 1). The results of univariate statistical analysis confirmed that statistically significant prognostic parameters included the age of patients ($P=0.038$), clinical stage ($P=0.001$), nuclear grade ($P=0.002$), depth of tumor invasion ($P<0.001$), and lymph node metastasis ($P=0.001$) (Table 2). On the other hand,

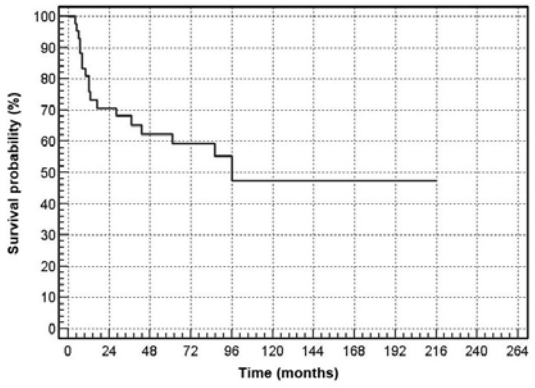

Figure 1. Overall survival in patients with invasive squamous cell carcinoma of the vulva (n=43).

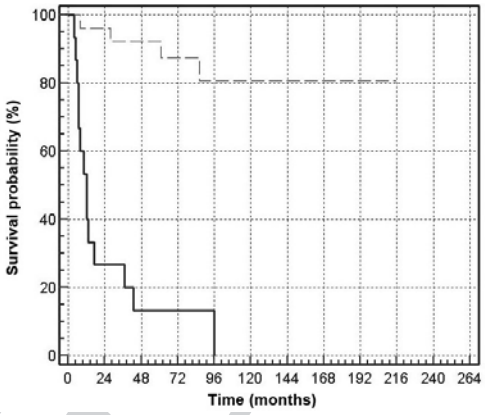

Figure 2. Overall survival in 43 patients with squamous cell carcinoma of the vulva according to the depth of tumor invasion: group with favorable prognosis – depth of invasion 5 mm or less (dashed line, n=28, 5-year survival 87.3%), group with unfavorable prognosis – depth of invasion deeper than 5 mm (full line, n=15, 5-year survival 13.3%).

the results of multivariate statistical analysis showed that only the depth of tumor invasion ($P<0.001$) can be considered an independent, statistically significant prognostic parameter (Fig. 2). Flow cytometric cell cycle parameters and mode of therapy had no impact on overall survival (Table 2, see also Fig. 2).

Discussion

The standard primary treatment for vulvar carcinoma is radical vulvectomy with bilateral inguinal lymphadenectomy. Surgery alone has sufficed for low-risk patients whereas the addition of groin and pelvic irradiation or chemotherapy has been of benefit for high-risk patients (14,17). This mode of therapy improves patients' survival and cure rates, but at the same time causes significant morbidity, such as wound breakdown, chronic leg edema, genital prolapse, and vaginal strictures (11). Therefore, we tried to identify a subgroup of patient population with favorable prognosis who may be candidates for trials on less aggressive mode of treatment. Superficially invasive squamous cell carcinoma was not included in this study, because this group of patients has a favorable prognosis with a less aggressive mode of treatment (1,2,11). In fact, we wanted to evaluate the importance of several prognostic parameters in the group of patients with deeper tumor invasion, because of well known association between the depth of invasion and lymph node involvement, recurrence, and survival.

According to the literature (1-2,6,14,24), prognosis is largely determined by the extent of

Table 2. Univariate and multivariate analysis of prognostic parameters in patients with invasive squamous cell carcinoma of the vulva (n=43)

Patient characteristics	Univariate statistics*				Multivariate statistics*			
	b	SE (b)	*P*	OR (95% CI)	b	SE (b)	*P*	OR (95% CI)
Age	1.18	0.57	0.038	3.25 (1.07-9.84)	0.52	0.61	0.386	
Menstrual status	-13.27	263	0.960					
Clinical stage	2.04	0.64	0.001	7.68 (2.22-26.56)	0.98	0.72	0.174	
Diameter of tumor	1.47	1.03	0.153					
Clitoral involvement	-0.36	0.31	0.253					
Histological grade	-0.94	0.49	0.055					
Nuclear grade	-2.36	0.76	0.002	10.64 (2.43-46.47)	-0.98	0.94	0.297	
Depth of invasion	-2.59	0.58	<0.001	14.26 (4.35-50)	-2.59	0.58	<0.001	14.26 (4.35-50)
Tumor growth pattern	0.22	0.57	0.702					
Lymph node metastasis	2.04	0.64	0.001	7.68 (2.22-26.56)	0.98	0.72	0.174	
DNA ploidy	0.01	0.53	0.982					
Proliferative activity†	0.78	0.59	0.186					
Therapy	0.76	0.49	0.120					

*Multivariate Cox regression analysis was performed only for parameters with significant ($P<0.05$) univariate impact. Considering the similar distribution of patients with respect to clinical stage and lymph node metastasis, multivariate analysis was only done for one of those parameters. SE – standard error; OR – odds ratio; CI – confidence interval.
†Unfavorable findings were tumors with more than 10% of cells in the $S+G_2/M$ cell cycle phases.

disease at the time of diagnosis as reflected by the association of outcome with lymph node status, tumor diameter, and depth of invasion. Although a variety of prognostic parameters have been proposed to supplement the clinical stage of disease, none has found a universal acceptance. In our study, the prognostic factors associated with overall survival in the group of patients with invasive squamous cell carcinoma of the vulva were age of patients, clinical stage, nuclear grade, depth of tumor invasion, and lymph node metastasis. Clinical stage was not found to be prognostically important in multivariate analysis. The explanation could be the exclusion of superficially invasive carcinoma from our study and at the same time, insufficient clinical evaluation of metastatic disease in the past, due to lack of modern imaging techniques. Additionally, the microscopic evaluation of lymph nodes for detection of metastatic cell squamous carcinoma may be augmented by immunohistochemistry using a polyclonal keratin antibody (25), which was not done in our study. All these circumstances could be the explanation why clinical stage was not shown to be an important independent prognostic factor in our group of patients.

Histopathological parameters were reviewed in all cases and histological grade was evaluated independently of nuclear grade. Interestingly, histological grade was not as important as nuclear grade. Our definition of grading is different than grading system used by Boyce et al (14), and this can explain different results. The American Joint Committee on Cancer (AJCC) recommends histopathological grading, but our results suggest that a multicenter study would be indispensable for the evaluation of the real meaning of nuclear grading.

The significance of depth of tumor invasion is most obvious in the example of superficially invasive vulvar cancer. For patients with this stage of the disease, the recommended therapy is wide local excision without vulvectomy. The probability of node metastasis in these patients is extremely small, so the current treatment is local excision without lymphadenectomy. In two small studies (26,27) specifically examining tumors with invasion of 1-2 mm, without vascular space involvement, none had node metastasis. A 3 mm depth of invasion has been found to be associated with inguinal lymph node metastasis in approximately 10%, whereas in a study of patients with vulvar squamous carcinoma with 5 mm depth of invasion, approximately 15% of women had inguinal lymph node metastasis (1). In our study, the depth of stromal invasion ≤5 mm was favorable prognostic parameter. It correlated most closely with survival and was the only factor that independently correlated with outcome in a multivariate analysis.

It has been shown that flow cytometric analysis of tumor cells (DNA ploidy and proliferative activity) is of prognostic value for patients with neoplasms of the uterine corpus (18-21), but the studies investigating prognostic value of DNA content in patients with vulvar carcinoma have been uncommon and have provided divergent results. DNA ploidy has been found to be of prognostic importance in some studies (8,9), but not in others (6,10,11). The influence of DNA ploidy and proliferative activity determined by flow cytometry was not shown as a potentially significant predictor of outcome in our study. The majority of tumors consisted of cells with diploid DNA (approximately 70%) and low proliferative activity, and these data are comparable with some other studies (10). The data were analyzed using several cutoff points for analysis of the proliferation index, ie percentage $S+G_2M$ phase <10, 10-15, 15-20, >20%. None of these subgroups appeared to reliably predict overall survival.

All of these prognostic factors were evaluated because we wanted to separate low-and high risk patients and suggest the best way of treatment planning. The undertreatment of high-risk patients is a problem, but at the same time, a considerable proportion of patients had been subjected to unnecessarily aggressive treatment regimens that probably contributed to increased morbidity or even mortality rates.

In conclusion, the age of patients at the time of diagnosis, clinical stage of the disease, nuclear grade, depth of tumor invasion, and lymph node metastasis are considered to be the parameters of crucial relevance for the prognosis of patients with squamous cell carcinoma of the vulva. However, only the depth of tumor invasion was proven to be independent statistically significant parameter relevant for the outcome of this disease. Tumor DNA ploidy and proliferative activity did not appear to be useful prognostic factors.

Acknowledgments

We thank Dr Mladen Petrovečki and Mr Marko Petrovečki for their help with statistical analysis of the data.

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Received: January 28, 2005
Accepted: March 21, 2005

Correspondence to:
Marin Nola
Department of Pathology
Zagreb University Hospital Center
Kišpatićeva 12
10000 Zagreb, Croatia
marin.nola@zg.htnet.hr