Prognostic significance of DNA cytometry in carcinoma of the uterine cervix FIGO stage IB and II

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Received November 2001
Accepted 18 November 2001

Objective: To assess the prognostic value of DNA-image cytometry in cervical carcinoma of the uterus and its relation to other established prognostic factors.

Study design: The study included 116 cases of cervical carcinoma FIGO stages IB and II which were treated with radical abdominal hysterectomy. The median follow-up was 55 months (range 1–162 months). DNA image cytometry was performed on cytologic specimens prepared by enzymatic cell separation from formalin-fixed, paraffin-embedded tissues. DNA stemline ploidy, DNA stemline aneuploidy, 5c exceeding rate, 9c exceeding rate, 2c deviation index, and DNA malignancy grade were computed. DNA-variables as well as various clinical and histological variables were related to survival rates.

Results: In multivariate statistical analysis DNA stemline ploidy using 2.2c as a cut-off value and FIGO stage showed to be statistically significant available presurgery predictors of survival, whereas the postsurgical parameters lymphonodal status, tumor size and parametrial involvement were significantly correlated with survival. The synopsis of all parameters in a multivariate Cox model indicated that – with declining relevance – the number of positive pelvic lymph nodes, DNA stemline ploidy using a cut-off level at a modal value of 2.2c, largest pelvic lymph node, 5c exceeding rate, and ratio of carcinoma area to cervix area, were of predictive value for survival.

Conclusions: Our results suggest that prognostic information deducted from classical staging parameters is successfully complemented by DNA image cytometry which can be applied pretherapeutically.

Keywords: Cervical cancer, staging, DNA image cytometry, aneuploidy, prognosis, grading

1. Introduction

Accurate prediction of the biologic behavior of malignant neoplasms is important in helping to determine patient management and in designing therapeutic trials. Clinical staging of cervical carcinomas so far, has been the most important single parameter influencing choice of treatment and indicating outcome [4]. However, it has some shortcomings: clinical staging has failed to predict the surgical stage correctly in a significant number of cases. Discrepancies in at least $1/3$ of cases have been reported [26,28]. In addition, clinical staging allows significant variation in tumor volume within stages. Tumor volume of cervical carcinoma has been proposed to be even more important, both in early stage and in locally advanced stage [8].

The cytophotometric identification of DNA distribution abnormalities has been shown to be prognostically important in several human malignancies including, e.g., prostate, bladder, and breast cancer [1,3,34]. In squamous cell carcinoma of the cervix, the results have been conflicting. Some studies have found a prognostic impact of DNA ploidy and/or S-phase fraction [21,24, 33,35], while others were not able to confirm this [23, 37]. Most of these studies have been performed using DNA flow cytometry. Studies which applied DNA im-

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Analytical Cellular Pathology 23 (2001) 97–105
ISSN 0921-8912 / $8.00

age cytometry agreed concerning the prognostic value of DNA cytometry, but these studies originated mostly in the 1970s and 1980s [2,9,15]. Meanwhile major effort has been undertaken to standardize DNA image cytometry [6,14,16,17].

The aim of the present study was to evaluate the prognostic significance of standardized DNA-image-cytometry on biopsies of cervical carcinoma in comparison to several clinical and histological variables.

2. Material and methods

2.1. Patients

Among the patients with cervical cancer who were treated in the Department of Obstetrics and Gynecology, University of Graz, Austria, from January 1977 to December 1988, 116 patients with primary invasive cervical carcinoma in FIGO Stages IB, IIA, and IIB were eligible for this study because they met the following conditions: a biopsy containing tumor tissue was available before surgery, the primary treatment was radical abdominal hysterectomy with pelvic lymph node dissection, postsurgical staging and a complete clinical follow-up were documented, and survival status to the date of last follow-up (on June 30, 1991) was known. The mean age of the patients was 47.2 ± 10.4 years (SD). The median follow-up time was 55 months (range 1–162 months). Among 54 patients with recurrent disease, 43 died and 11 were still alive with tumors for which they were receiving palliative therapy at the closing date of the study. Five patients intercurrently died of a disease unrelated to cervical cancer. Postsurgical adjuvant treatment was not allowed for the estimation of survival rates.

2.2. Pathology

All biopsy specimens were recut from the paraffin-embedded tissue blocks for hematoxylin–eosin (H&E) staining and reexamined by one of the authors (A.B.). Histologic type and grade was redetermined according to the World Health Organization classification [32]. Mitotic rate, lymphovascular space involvement, carcinoma area, cervix area, ratio of carcinoma area to cervix area, microscopic evidence of parametrial involvement, number of dissected parametrial lymph nodes, number of pelvic lymph node metastases, and size of largest pelvic lymph node were retrieved from the original pathology reports.

2.3. Image cytometry

Those areas of the H&E-stained sections of the biopsy specimens which were relevant for the histologic diagnosis were marked in order to prepare tumor cell monolayer smears by a cell separation technique described elsewhere in detail [6]. In brief, from the area of the tissue block corresponding to the marked area of the H&E-stained section, three 50 μm-thick sections were cut and placed in a glass tube. The material was deparaffinized with xylene, rehydrated and washed in a phosphate-buffered solution (PBS) (pH 7.2). Afterwards it was suspended in 0.5% pepsin in 0.2% HCl and incubated at 37°C for 30 minutes. The pepsinized material was then centrifuged and mechanically dispersed with an Ultra-Turrax homogenizer (Janke & Kunkel, Staufen, Germany) using 10,000 rpm for 30 seconds. After mechanical separation the suspension was filtered through a 70 μm nylon mesh, centrifuged and smeared on poly-L-lysine-coated slides.

Feulgen staining was performed automatically in a modified staining machine, Varistain-24 (Shandon, Pittsburgh, Pennsylvania, USA), as described previously [11]. In brief, acid hydrolysis (4 N HCl, 27.5°C, 55 minutes) was followed by 60-minute incubation in Schiff's reagent (Merck, Darmstadt, Germany) at room temperature.

Measurements were performed with a TV image analysis system, the MIAMED-DNA (Leitz, Wetzlar, Germany) combined with an automated microscope. Details of the measurement procedure were described by Sanchez et al. [31]. The configuration included a Saticon 1″ TV-camera (Bosch, Stuttgart, Germany), a stabilized power supply for a 12 V halogen lamp, and a 570 nm-interference filter with a halfwidth of 10 nm. Measurements were performed using a 40/0.70 objective. In each case 200 tumor cells were measured at random. At least 20 lymphocytes were measured as reference cells for DNA parameters; the mean of their integrated optical density was multiplied by a correction factor of 1.25 to obtain the normal 2c value. The coefficient of variation (CV) of the reference cell populations was below 5%.

The following parameters were calculated:

- DNA stemline ploidy:
  DNA stemline ploidy was defined as the modal value of a DNA stemline [17]. Two subgroups of DNA stemline ploidy were differentiated using a cut-off level at a modal value of 2.2c.

– DNA stemline aneuploidy:
  DNA stemline aneuploidy was assumed, if the modal value of a DNA stemline was <1.80c and >2.20c or <3.60c and >4.40c [16].
– The 5c exceeding rate (5cER) and the 9c exceeding rate (9cER):
  are defined as the percentage of aneuploid cells having a DNA content of more than 5c or 9c, respectively [5]; DNA aneuploidy was detected by a single-cell interpretion if at least three 5c-exceeding events (5cEE) and/or at least three 9c-exceeding events (9cEE) [10] were found.
– The 2c deviation index (2cDI):
  defined as the mean square deviation of n measured tumor cells, c_i, from the diploid value representing the variance of the tumor cell population around the normal 2c value [16]:

$$2\text{cDI} = \frac{1}{n} \times \sum_{i=1}^{n} (c_i - 2\text{c})^2.$$

– The DNA malignancy grade:
  was calculated from the 2cDI value as previously described [5].

2.4. Statistical analysis

Overall survival time was calculated from the date of surgery to the date of last follow-up or death caused or accompanied by clinical evidence of cervical carcinoma. Patients dying from causes unrelated to cervical cancer were censored at the time of death. The Kaplan–Meier method was used to estimate the survival rate and survival curve in each parameter, and the differences between curves were assessed using the Wilcoxon–Breslow test. Statistical significance of univariate analysis was defined as $P < 0.05$. Cox's proportional hazards model was used to evaluate the prognostic power of various factors in multivariate analysis. All of the statistical calculations were done using BMDP software package (Department of Biomathematics, University of California, Los Angeles, USA) [12]. This software package used $P > 0.1$ as a cut-off value for the exclusion of a variable from the Cox model.

3. Results

The DNA cytometric, clinical, and histological parameters which were significantly correlated with survival are summarized in the first column of Table 1. The availability of these prognostic factors either before or after surgery is indicated. For statistical analysis 116 patients were stratified by FIGO stage. When the strata differed with respect to the characteristic under study they were placed on top of each other, otherwise next to each other (column 2). Subgroups in which the feature under study did not show a significant statistical correlation with survival were for the most part omitted from this table to simplify the presentation. The number of patients within the subgroups is given in column 3. The prognostic implication of a characteristic is listed in the fourth column. Finally, the last column shows the p-value of the respective prognostic statement. If necessary, the strata in which the prognostic value of a particular parameter was tested and the respective p-values are marked, e.g., with an asterisk.

Presurgically 46 of the 116 patients were assigned to FIGO stage IB and 70 to stage II. All of the latter presented with a stage IIB-tumor with exception of one patient who revealed a stage IIA tumor. Out of 46 patients belonging to the FIGO stage IB-subgroup 13 (28.3%) had a recurrent disease and 11 of these (24.0%) died of cervical cancer. One died of a disease unrelated to her cervical carcinoma. In contrast 41 (58.6%) out of 70 patients with a FIGO stage II-tumor had a recurrent disease and 29 of these (41.4%) died of their cervical cancer. Within this subgroup an additional four patients died of unrelated disease. The different survival rates are illustrated in Fig. 1 by the Kaplan–Meier method and were statistically significant in univariate analysis ($p = 0.01$). The age of the patients did not correlate with the clinical outcome.

Like FIGO staging, the results of DNA image cytometry are available before surgery when the measurements are performed on biopsy specimens after enzymatic cell separation. In this study tumors with a DNA stemline ploidy $\leqslant$2.2c showed a significantly better survival than hyperdiploid tumors (Fig. 2, $p < 0.05$). The prognostic implication was valid both for patients with stage IB- and stage II-tumors. Four cases were excluded from the statistical analysis, because it was not possible to define a DNA stemline in the DNA histogram. The stratification into three DNA-ploidy-groups (<1.80c and >2.20c or <3.60c and >4.40c) according to Haroske et al. [16] did not discriminate statistically significant prognosis (data not shown). Nevertheless there was an obvious tendency for a better survival of patients with diploid tumors as compared to those with hyperdiploid tumors which might have become statistically significant in a larger

Table 1
Clinicopathologic and DNA image cytometric characteristics and their relation to survival (univariate analysis)

Characteristic	FIGO stage[a]	n	Prognosis	P
Presurgery				
FIGO stage				
Ib	Ib	46	favorable	0.01
II	II	70	unfavorable	
DNA stemline ploidy				
$\leqslant$2.20	Ib, II	24	favorable	$<$0.05
$>$2.20	Ib, II	88	unfavorable	
Postsurgery				
Carcinoma area $\leqslant$349 mm^2	Ib	23	favorable	$<$0.01
Carcinoma area/cervix area $\leqslant$0.36	Ib	23	favorable	$<$0.05
Lymphvessel invasion				
No	Ib	22	favorable	$<$0.05
Yes	Ib	24	unfavorable	
Histologic parametrial involvement				
No	Ib*	34	favorable	$<$0.001[b]
	II	43		
Yes	Ib**	12	unfavorable	
	II	27		
No. of parametrial lymph nodes				
$\leqslant$2	Ib, II	45		
3–5†	Ib, II	44		
$>$5‡	Ib, II	27	unfavorable	$<$0.01[b]
Pelvic lymph nodes				
Negative	Ib$^{\#}$	25	favorable	$<$0.05[b]
	II†	30		0.01[b]
Positive	Ib$^{\#\#}$	21	unfavorable	
	II‡	40		
No. of positive pelvic lymph nodes				
0$^{\#}$	Ib, II	55	favorable	$<$0.05[b]
$\leqslant$2$^{\#\#}$	Ib, II	35	unfavorable	0.01[b]
$>$2‡‡	Ib, II	26	unfavorable	
Largest positive pelvic lymph node				
$\leqslant$10 mm^2	II	16	unfavorable	$<$0.05
$>$10 mm^2	II	23		

[a] Statistical analysis compares subgroups stratified by FIGO stage.
[b] Significant differences between * and ** ($P < 0.001$), † and ‡ ($P < 0.01$), ## and ‡‡ ($P < 0.01$), and # and ## ($P < 0.05$).

study group (data not shown). The DNA proliferation fraction, the 5c and 9c exceeding rates, the 2c deviation index, and the DNA malignancy grade did not prove as valid predictors of survival in univariate analysis.

The histologic type of the tumors was predominantly squamous with 111 squamous cell carcinomas, four adenocarcinomas, and one adenosquamous carcinoma. The histologic type failed to show any statistically significant relation to survival. But there was a tendency of unfavorable clinical outcome in cases with small cell non keratinizing squamous cell carcinoma which might have been statistically significant in a larger study group. No correlation with clinical outcome was found for the mitotic rate.

Postsurgical histological parameters available were retrieved from the original pathology reports. Among them, tumor size turned out to be a prognostically significant parameter only in patients without clinically diagnosed parametrial involvement (FIGO stage IB). In this study small tumors with a carcinoma area

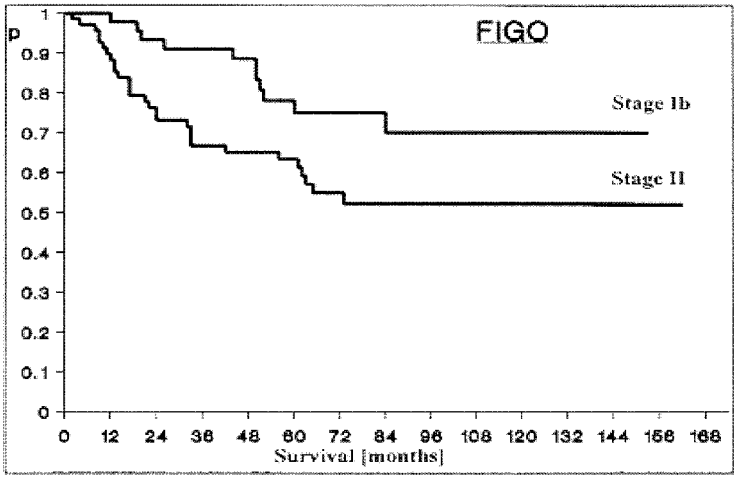

Fig. 1. Time of survival (months) correlated with clinical stage according to FIGO. The two groups constitute 46 (stage IB) and 70 (stage II) patients, respectively. $P = 0.01$.

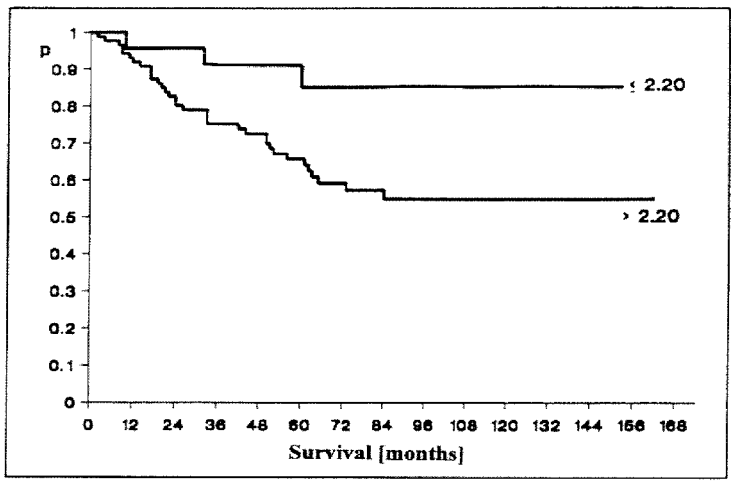

Fig. 2. Survival in months according to DNA ploidy using 2.2c as a cut-off value ($P < 0.05$). The two groups constitute 24 ($\leqslant$2.2c) and 88 ($>$2.2c) patients, respectively.

$\leqslant$349 mm^2 ($p < 0.01$) or a carcinoma area/cervix area-ratio $\leqslant$0.36 ($p < 0.05$) were correlated with a favorable clinical outcome on condition that the tumor belongs to FIGO stage IB. Tumor size was no longer prognostically relevant in patients with a stage II-tumor (data not shown).

Like tumor size, lymphvessel invasion proved to be a prognostically valid parameter only in patients without clinically diagnosed parametrial involvement (FIGO stage IB). In these patients evidence of lymphvessel invasion was an indicator of poor clinical outcome ($p < 0.05$).

Parametrial involvement is a parameter which enters into the FIGO-staging system. Table 1 shows that the correlation of clinical and histological evaluation of parametrial involvement is poor. In only 61 out of 116 patients (52.6%) the clinical assessment of parametrial involvement performed presurgically was in accordance with postsurgical histopathologic examination. Patients who were assigned to FIGO stage IB and turned out to have histologic parametrial involvement showed an unfavorable prognosis ($p < 0.001$). Interestingly enough, the lacking histologic proof of parametrial involvement did not influence the survival of patients with a tumor clinically estimated at stage II.

Both patients with stage IB- and stage II-tumors showed an unfavorable prognosis if a high number ($>$5) of parametrial lymph nodes was dissected

($p < 0.01$). There was no correlation between clinical outcome and dissection of 5 or less parametrial lymph nodes. The presence of carcinomatous involvement of the dissected parametrial lymph nodes was not investigated in this study.

The evidence of pelvic lymph node metastases and its number was of prognostic significance both for stage IB- and II-tumors. The results of this study show that tumorous lymph nodes are a frequent finding in both stages and that this is followed by a deteriorated clinical outcome (stage IB: $p < 0.05$; stage II: $p < 0.01$). Twenty-one (45.7%) out of 46 tumors in stage IB and 40 (57.1%) out of 70 tumors in stage II had pelvic lymph node involvement. In addition, the detection of more than two pelvic lymph node metastases resulted in an even more unfavorable prognosis ($p < 0.01$).

Finally, in stage II-tumors the size of the largest pelvic lymph node was relevant for the prognosis. Patients with a pelvic lymph node >10 mm^2 had an unfavorable clinical outcome as compared with those with a largest pelvic lymph node $\leqslant 10$ mm^2 ($p < 0.05$).

Multiparameter analysis. Cox stepwise regression analysis of the presurgical parameters revealed FIGO stage, DNA stemline ploidy using 2.2c as a cut-off value, 5cER, and mitotic rate as independent predictors of survival (Table 2). The multivariate analysis of postsurgery parameters available showed that the number of tumorous pelvic lymph nodes, the size of the biggest pelvic lymph node, and the ratio of carcinoma area to cervix area were prognostically significant (data not shown). Finally, Cox stepwise regression analysis of all investigated parameters revealed the number of positive pelvic lymph nodes, DNA stemline ploidy using a cut-off level at a modal value of 2.2c, largest pelvic lymph node, 5c exceeding rate, carcinoma area/cervix area-ratio, and DNA-proliferation fraction to be of independent prognostic significance with declining relevance (Table 3). In contrast FIGO stage did not retain independent prognostic significance after inclusion of the postsurgery available parameters into the Cox model.

For validation of the Cox model, the individual risk of the patients resulting from their individual profile of prognostically relevant factors and regression coefficients was calculated by a risk function: $R = \mathrm{EXP}(1.1906\,\mathrm{RCC} + 0.1106\,\mathrm{NLN} + 0.0434\,\mathrm{SLN} + 0.7599\,\mathrm{STL} + (-0.0343\,\mathrm{5cER}))$. The cutpoints of prognostically relevant factors used were 0.31 and 0.74 for RCC (ratio of carcinoma area to cervix area), 0 and 2 for NLN (number of pelvic lymph node metastases), 10 mm^2 for SLN (size of the largest pelvic lymph node), 2.2c for STL (DNA stemline ploidy), and 7.05 and 22.05 for 5cER (5c exceeding rate). According to the quantile of the distribution three risk groups were formulated (RI: $n = 28$; RII: $n = 56$; RIII: $n = 28$). The median survival time differed significantly between the three groups (RI vs. RII: $p < 0.05$; RII vs. RIII: $p < 0.001$) and is documented in Fig. 3.

Table 2
Presurgery available prognostic factors in multivariate analysis (Cox proportional hazards regression model)

Variable	Improvement		Global	
	Chi-square	P value	Chi-square	P value
FIGO stage	4.95	0.026	4.71	0.030
DNA stemline ploidy	3.15	0.076	7.68	0.022
5cER	7.02	0.008	13.61	0.003
Mitotic rate	3.98	0.046	18.06	0.001

Table 3
Postsurgery synopsis of prognostic factors in multivariate analysis (Cox proportional hazards regression model)

Prognostic variable	Improvement		Global	
	Chi-square	P value	Chi-square	P value
No. of positive pelvic lymph nodes	15.88	0.000	25.11	0.000
DNA stemline ploidy	3.51	0.061	28.30	0.000
Largest pelvic lymph node	3.28	0.070	31.59	0.000
5cER	3.51	0.061	32.60	0.000
Carcinoma area/cervix area	3.21	0.073	34.62	0.000

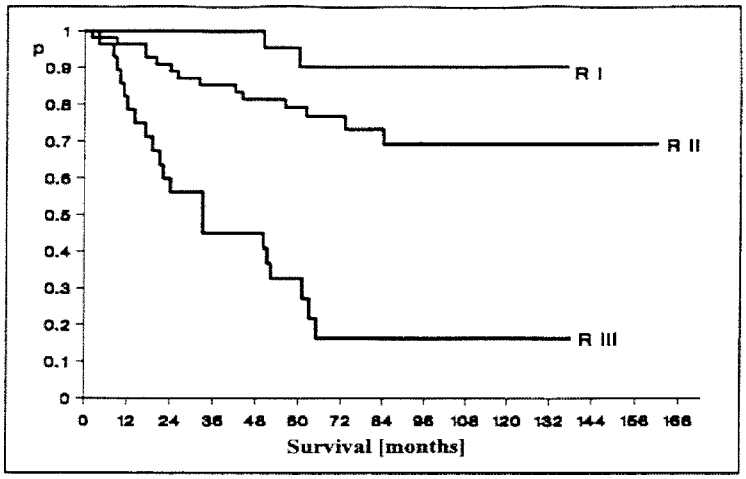

Fig. 3. Survival rates by risk groups. Significantly better survival was in the low risk RI-subgroup ($n = 28$) as compared to the intermediate risk RII-subgroup ($n = 56$), and in the intermediate risk RII-subgroup as compared to the high risk RIII-subgroup ($n = 28$) ($P < 0.05$ and $P < 0.001$, respectively).

4. Discussion

Although there has been considerable interest in diagnostic DNA cytometry for an objective assessment of tumor prognosis, its utility for patients with cancer of the cervix is still in question. Jakobsen and coworkers [21] and Lai et al. [24] showed that aneuploid tumors with a high relative DNA content were associated with a poorer prognosis. The Scandinavian study [21] connected a DNA index above 1.5 with an unfavorable outcome. Lai et al. [24] suggested a DNA index of 1.3 as a cut-off value. In contrast to this, Atkin and colleagues [2] and Rutgers et al. [30] found that DNA peridiploid tumors were most aggressive. Two prospective studies concerning the prognostic information of DNA flow cytometry in cervical squamous cell carcinoma linked a high S-phase fraction (SPF) in near-diploid tumors with an unfavorable outcome [33, 35]. However, DNA ploidy level had no prognostic significance when SPF was not included in the multivariate analysis. Other authors have failed to find any correlation between DNA ploidy or SPF and survival [23, 37].

In this study, the prognostic value of several variables measured by DNA-image-cytometry was compared with comprehensive information about clinical and histopathological variables. All biopsy specimens analyzed originated from patients with cervical carcinoma treated with radical abdominal hysterectomy and bilateral pelvic lymphadenectomy. The results showed that a DNA stemline ploidy above 2.2c was significantly related to survival in univariate analysis and that it was the second most relevant prognostic factor both presurgically and postsurgically in multivariate analysis. Before surgery, its prognostic significance was only surpassed by clinical stage. After hysterectomy and pelvic lymph node dissection, solely the number of pelvic lymph node metastases achieved more important prognostic significance than DNA-stemline ploidy whereas FIGO stage lost its independent prognostic value in multivariate analysis.

DNA image cytometry and flow cytometry are objective methods to study abnormalities of nuclear DNA distribution. However, because of the different methods of preparation of nuclear suspensions and histogram interpretation, substantial subjectivity existed so far especially with regard to older studies. Lacking standardization may partially explain why the published rates of non-diploid tumors (e.g., Lai et al. [24], Jakobsen [20]) in cervical carcinoma vary widely (35–80%) and is likely to have had a major impact on the prognostic validity of diagnostic DNA cytometry in the past. In the present study 78% of tumors revealed DNA-stemlines above the cut-off value of 2.2c. In 1995 a European consensus report on standardization of diagnostic DNA image cytometry was presented [7] to overcome the problem of lacking standardization and has since then been updated twice [14,16,17]. To the best of our knowledge all data available in literature on the prognostic significance of DNA image cytometry in cervical squamous cell carcinoma were generated before 1995 [2,9,15,25].

The majority of investigations on prognostic value of DNA cytometric analysis have been performed by

flow cytometry [21,23,24,30,33,35,37]. Still, disadvantages of flow cytometry are its inability to measure mixed populations separately along with the lacking visual control of cells under investigation [6]. In addition, flow cytometric studies frequently do not apply an internal or external standard. Many investigators assigned a diploid DNA amount to the histogram peak revealing lowest DNA content, even if it might have represented a hypo- or hyperdiploid DNA stemline. Consequently the lack of an internal reference may result in overlooking hypo- or hyperdiploid (peridiploid), but nevertheless aneuploid tumor cell populations.

Another possible explanation for the indecision regarding the usefulness of DNA cytometry in determinating the prognosis of patients with cervical cancer is that the choice of therapeutic regimen interferes with the survival of patients with aneuploid tumors and/or tumors with high SPF. Several authors suggested that DNA-aneuploid tumors may be more sensitive to radiation therapy [13,25,36]. The improved response rate in DNA-aneuploid tumors is likely to be related to the higher percentage of cells in the S + G2/M phases of the cell cycle, since the killing effect of radiation is cell-cycle dependent and aneuploid tumors often have a higher proliferative rate compared to diploid tumors. Atkin [2] and Rutgers et al. [30] who connected non-diploid tumors to a favorable prognosis exclusively investigated patients who underwent primary radiation therapy. On the other hand, Naus and Zimmerman [27], Willén et al. [35], and Kristensen et al. [23] investigated a heterogenous population in regard to the therapeutic regimen. They stated that their data did not demonstrate any differences between surgical or radiation therapy alone.

The 5c exceeding rate (5cER) is another variable that was found to be correlated with survival in multivariate analysis in this study. 5cER is defined as the percentage of cells exceeding a DNA content of 5c and has been used for the grading of malignancy [5]. The 9c exceeding rate, 2c deviation index, and DNA malignancy grade had no prognostic significance.

Beside the measurements by DNA image cytometry the present study compiled data on several clinical and histopathological variables. Like other authors we found clinical stage and number of pelvic lymph node metastases to be the most important prognostic factors presurgically and postsurgically, respectively [4,19]. However, the present study confirms the observation [26,28] that presurgical clinical staging fails to predict parametrial involvement in a significant number of cases correctly. In only 54% of the cases FIGO stage was in accordance with postsurgical histopathologic examination of parametrial involvement (Table 1).

The prognostic significance of tumor size, ratio of carcinoma area to cervix area, lymph vessel invasion, mitotic rate, parametrial invasion, number of parametrial lymph nodes, and largest pelvic lymph node is in accordance with other studies [4,18,22]. Age, histologic type, and grade have been associated with survival, but a review of the literature shows contradictory view-points [4,29]. The present study did not reveal such statistically significant correlations.

In conclusion, the results of this study confirm the prognostic value of standardized DNA image cytometry and suggest that a DNA stemline ploidy above 2.2c is correlated with an unfavorable prognosis of cervical carcinoma. Therefore, the assessment of DNA stemline ploidy could lead to a better definition of subsets of patients requiring a distinctive treatment.

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