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Prognostic significance of preoperative DNA flow cytometry in surgically-treated cervical cancer

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Summary

Objective: To evaluate the prognostic significance of preoperative DNA flow cytometry compared with other clinical and histologic variables in cervical carcinoma.

Study design: Sixty-four patients with FIGO Stage Ib-III cervical cancer treated with radical abdominal hysterectomy and systematic pelvic lymphadenectomy were analyzed. The mean follow-up was 3.4 (range 0.3-9.8) years. DNA flow cytometry was performed with fresh tumor tissue. Four biopsies were recut from the surgical specimen within 30 minutes of the operation. The ectocervix was divided into four quadrants and a specimen obtained from each. DNA-low-grade tumors (diploid, near-diploid, tetraploid and near-tetraploid) were distinguished from DNA-high-grade tumors (aneuploid and hypoploid). Carcinomas with more than one non-diploid stem line were considered heterogeneous. An S phase fraction >7% was classified as low, 7% - < 14% as moderate, and ≥14 as high. DNA ploidy, DNA heterogeneity, S phase fraction and various clinical and histological variables were related to disease-free survival.

Results: In the univariate analysis patients with DNA-low-grade carcinomas had significantly better disease-free survival than patients with DNA-high-grade tumors (82% vs 45%, p = 0.021). Carcinomas with an S-phase fraction < 7% were associated with better disease-free survival (0.8) than those with an S-phase fraction 7% - > 14% (0.62) and those with ≥ 14% (0.64), but this was not statistically significant. Cox stepwise regression analysis showed DNA-heterogeneity, age, grade, parametrial involvement and extrapelvic metastasis to be independent prognostic factors.

Conclusion: DNA ploidy and DNA heterogeneity are of prognostic importance in cervical cancer. DNA flow cytometry may be used preoperatively to identify low-risk and high-risk patients within a given stage.

Key words: Cervical cancer; Staging; DNA flow cytometry; Aneuploidy; Prognosis.

Introduction

Most prognostic factors in patients with cervical carcinoma (i.e., lymph node status) are post-therapeutic. An assessment of the anatomic extent of the disease, as indicated by the FIGO stage, is available before treatment, but clinical staging often incorrectly predicts the surgical stage [27, 29]. Also, the clinical stage is a rather poor indicator because a given clinical stage, such as IB, includes a wide range of tumor volumes. DNA distribution abnormalities have been identified as prognostically important in several human malignancies [3, 6]. Because the uterine cervix is accessible such abnormalities can be measured before treatment in most tumors. The aim of the present study was to evaluate the prognostic significance of DNA flow cytometry compared with other clinical and histologic variables in cervical carcinoma.

Material and Methods

Patients

Sixty-four patients with FIGO Stage Ib-III cervical cancer treated at our institution between January 1994 and December 2000 were eligible for this study because they met the following criteria: Multiple biopsies of the tumor were available, the primary treatment was radical abdominal hysterectomy with systematic pelvic lymphadenectomy [35], postsurgical staging was performed and a complete follow-up up to June 30, 2001 was known. The mean age of the patients was 45 (range 19-76) years. The mean follow-up was 3.4 (range 0.3-9.8) years. After primary surgery 35 patients (55%) with risk factors (parametrial involvement, lymph node metastasis, positive surgical margins) received pelvic irradiation (50 Gy) or cytotoxic chemotherapy with six courses of cisplatin (50 mg/m^2) or carboplatin (400 mg/m^2) and bleomycin (30 mg). Patients were seen every three months during the first year after surgery and every 6-12 months thereafter. Twenty patients developed recurrent disease, 13 of whom died and seven of whom are alive. Two patients not included in the study died of causes unrelated to cervical cancer.

Pathology

Four biopsies of fresh tumor tissue were recut from the surgical specimen within 30 minutes of the operation. The ectocervix was divided into four quadrants and a specimen obtained from each, and the specimens were then halved. One half was frozen at –70 °C, the other was fixated in formalin, embedded in paraffin, and stained with HE. Histologic type and microscopic grade of malignancy of adenosquamous carcinomas and adenocarcinomas were determined according to the World Health Organization classification [31]. A modification of the Broder method [7] was used for microscopic grading of squamous cell carcinomas. In well differentiated (grade 1) tumors, the most striking feature is abundant keratin, which is deposited as concentric

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whorls in the centers of neoplastic epithelial nests. The cells appear mature, with abundant eosinophilic cytoplasm. Dyskeratosis may be present. The cells have well-developed intercellular bridges. The nuclei are large, irregular und hyperchromatic. Mitotic figures are present. In moderately differentiated (grade 2) carcinomas, the neoplastic cells are more pleomorphic than in grade 1 tumors, characterized by large irregular nuclei, and have less abundant cytoplasm. The cellular borders, as well as intercellar bridges, appear indistinct. Keratin pearl formation is virtually nonexistent. Mitotic figures are more numerous than in grade 1 carcinomas. Poorly differentiated (grade 3) tumors are composed of cells with hyperchromatic oval nuclei and scant indistinct cytoplasm. Clear-cut squamous differentiation by keratinization may be difficult to find. Mitoses and areas of necrosis are abundant [36]. Involvement of the parametrial tissue and parametrial lymph nodes was studied in step serial frontal sections. The extirpated lymphatic fatty tissue underwent complete pathological processing. Histology showed 53 squamous cell carcinomas, seven adenosquamous carcinomas and four adenocarcinomas. Five tumors were well differentiated, 31 moderately differentiated and 28 poorly or undifferentiated.

DNA flow cytometry

Fresh tumor tissue was dissected with a scalpel and treated with 1 ml pepsin -HCl (pH 1) for about 10-15 min, depending on the texture of the sample. The samples were than stained with 4´,6-diamidino-2-phenylindole according to Goehde *et al.* [13] and analyzed with a PAS III cytometer (Partec, Münster, Germany) with multicycle software (Phenix Flow Systems, San Diego, CA, U.S.A.). Human lymphocytes were used for calibration. At least 20,000 nuclei were analyzed from each sample. The mean coefficient of variation of all G1 peaks was <3%. The following DNA-types were differentiated: diploid, near-diploid, tetraploid, near-tetraploid, aneuploid, and hypoploid (Table 1).

Table 1. — *DNA-ploidy of 64 cervical cancers.*

	DNA-index	N (%)
Diploid	0.97-1.03	9 (14)
Near-diploid	0.90 - <0.97 and >1.03-1.1	7 (11)
Tetraploid	1.94-2.06	7 (11)
Near-tetraploid	1.8 - <1.94 and >2.06-2.2	12 (19)
Aneuploid	>1.1 - <1.8 and >2.2	25 (39)
Hypoploid	<0.9	4 (6)

DNA-low-grade tumors (diploid, near-diploid, tetraploid and near-tetraploid carcinomas) were distinguished from DNA-high-grade tumors (aneuploid and hypoploid carcinomas). If a tumor contained more than one cell line, the case was classified on the basis of the sample with the largest deviation in the following order: diploid, near-diploid, tetraploid, near-tetraploid, aneuploid or hypoploid. Diploid carcinomas and carcinomas with one non-diploid stem line were considered homogeneous. Carcinomas with more than one non-diploid stem line were considered heterogeneous. A S-phase fraction < 7% was classified as low, 7% - < 14% as moderate, and ≥ 14 as high.

Statistical analysis

FIGO stage, age, grade of differentiation, DNA ploidy, DNA heterogeneity, S phase fraction, parametrial involvement, status of the lymph nodes, and extrapelvic metastases were compaired with the Chi-square test and Fisher's exact test. Disease-free survival (DFS) was defined as the period between primary surgery and death or relapse. Survival analysis was done with the Kaplan-Meier estimator and the log-rank test. Cox proportional-hazards regression model was used to evaluate the simultaneous effect of the co-variables, p values < 0.05 were considered statistically significant. Data were analyzed with SPSS 10.0 (©SPSS Inc, Chicago, Illinois, U.S.A.), SAS 8.00 (©SAS Institute Inc., Cary, NC, U.S.A.), and StatXact (Cytel) software.

Results

Nine carcinomas (14%) were diploid and 55 (86%) were non-diploid. Forty-two (66%) of the 64 carcinomas showed DNA heterogeneity. Ten (16%) carcinomas had low, 11 (17%) had moderate and 43 (67%) had high S phase fraction. Thirty-five (55%) carcinomas were classified as DNA-low-grade whereas 29 (45%) were classified as DNA-high-grade. Thirty-three carcinomas (51%) showed parametrial involvement. Twenty-five patients (39%) had positive pelvic lymph nodes and eight patients (12%) had extrapelvic metastases (Table 2).

Table 2. — *Clinicopathologic and DNA-flow cytometric characteristics and their relation to disease-free survival (univariate and multivariate analysis).*

Characteristic	n	Recurence (n)	Disease free survival	p univariat	p-Cox mutivariat
FIGO stage				0.0023	0.095
IB	31	4	0.859		
II	28	13	0.494		
III	5	3	0.4		
Age				0.065	0.226
<40	22	8	0.654		
40 - <50	23	3	0.856		
≥ 50	19	9	0.486		
Grade of differentiantion				0.6596	0.033
Well	6	1	0.833		
Moderate	30	10	0.621		
Poor	28	9	0.673		
DNA Ploidy				0.0215	0.345
DNA-low-grade	35	7	0.825		
DNA-high-grade	29	13	0.451		
DNA Heterogeneity				0.6249	0.018
No	22	6	0.765		
Yes	42	14	0.614		
S phase fraction				0.617	0.521
<7%	10	2	0.8		
7% - <14%	11	4	0.623		
≥14%	43	14	0.649		
Parametrial involement				0.0000	0.013
No	31	2	0.927		
Yes	33	18	0.447		
Lymph node metastases				0.1049	0.924
No	39	9	0.729		
Yes	25	11	0.573		
Extrapelvic metastases				0.0003	0.075
No	56	13	0.763		
Yes	8	7	0.125		

Univariate analysis: The flow-cytometric, clinical, and histologic parameters and their relation to DFS are summarized in Table 2. FIGO stage, parametrial involvement and extrapelvic metastases correlated significantely with DSF. Patients with DNA-low-grade carcinomas had a significantly better DSF than patients with DNA-high-grade tumors (82% vs 45%, p = 0.021, Figure 1). The stratification into three S-phase-fraction groups did not prove a valid predictor (Table 2).

Multivariate analysis: Cox stepwise regression analysis revealed DNA-heterogeneity, age, grade, parametrial involvement, distant metastasis as independent predictors of disease-free survival (Table 2).

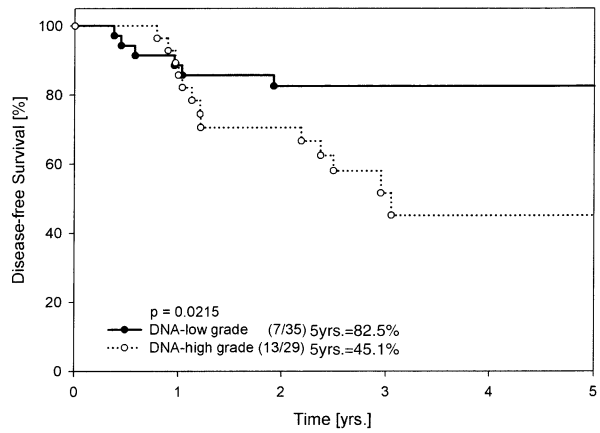

Figure 1. — Disease-free survival in 64 cervical cancers.

Discussion

In this prospective study 86% of cervical carcinomas had abnormalities of nuclear DNA distribution by DNA flow cytometry. We discriminated between DNA-low-grade and high-grade carcinomas as well as between carcinomas with and without DNA heterogeneity because tumorogenesis is a multistep, multigenetic process and in cytogenetic tumor progression sequential effects are often observed:

1) DNA ploidy decreases or increases slightly to below or above a DNA index of 1.0 (diploid, near-diploid). This is caused by net losses or gains of chromosomes or parts of them.

2) First aneuploid polyploidization of the diploid or near-diploid tumor stem line, resulting in a second stem line near a DNA index of 2.0 (tetraploid, near-tetraploid) with doubled DNA content.

3) Stemline ploidies decrease by loss of chromosomes in polyploized tumor cells (aneuploid, hypoploid).

4) Repeated polyploidization and/or further losses of chromosomes results in advanced genetic instability (aneuploid, hypoploid) (6,20).

Fifty-five percent of cervical carcinomas were DNA-low-grade while 34% of carcinomas showed DNA homogeneity (Table 2). Tumors in which only minor DNA distribution abnormalities were detectable and carcinomas with DNA homogeneity may represent early phases of cytogenetic tumor progression and genetic stability. In contrast DNA-high-grade carcinomas (45%) with major DNA distribution abnormalities and carcinomas with DNA-heterogeneity (66%) may indicate late phases of cytogenetic tumor progression and genetic instability.

In this study DNA ploidy and DNA heterogeneity had prognostic importance. In the univariate analysis patients with DNA-low-grade carcinomas had significantly better DFS than patients with DNA-high-grade tumors (82% vs 45%, p = 0.021) (Figure 1). The synopsis of DNA flow cytometric, clinical and histological parameters in a multivariate Cox model indicated that DNA heterogeneity is an independent prognostic factor (p = 0.018) (Table 1). Based on these results, DNA-high-grade cervical cancers and tumors with DNA heterogeneity may warrant more aggressive therapy, even in the absence of morphological risk factors. These results are consistent with reports that the more malignant the tumor, the higher and more widely dispersed the DNA content of the tumor cell nuclei [4] and with the results based on standarized DNA image cytometry of cervical carcinomas. Grote *et al.* [15] reported 116 patients with cervical carcinoma FIGO Stage IB and II. Stemline ploidy above 2.2 c was associated with an unfavorable prognosis and the 5 c exceeding rate was found to be relevant for survival in multivariate analysis.

In this study carcinomas with an S-phase fraction < 7% were associated with better DSF (0.8) than carcinomas with an S-phase fraction 7% - < 14% (0.62) and those with ≥14% (0.64) but this was not statistically significant (Table 1). In a larger study of 307 patients Strang *et al.* [32] reported S-phase fraction to be significant for survival, but in cervical cancer the significance of S-phase fraction is controversial [9]. A study of mitotic index failed to demonstrate a relationship with survival in an univariate analysis [16].

In 1959 Atkin *et al.* [2] were the first to correlate the distribution of nuclear DNA in cervical carcinomas with patient survival, but subsequent reports on DNA cytometry in cervical carcinomas have been conflicting. A number of studies have shown that DNA distribution abnormalities correlate with the prognosis in cervical carcinoma [1, 3, 5, 8, 11, 14, 17-19, 22-24, 28, 30, 32, 34) but others reported no prognostic value [9, 10, 21, 26, 33, 38]. These discrepancies are not surprising. In contrast to DNA image cytometry, DNA flow cytometery is still not standardized and different studies used different definitions, methods, standards and interpretations, were retrospective [10, 14, 17-19, 22-24, 26, 28, 30, 33, 38], used paraffin-embedded material [10, 17-19, 22-24, 26, 28, 30, 32-34, 38], and based results on only one tumor sample [10, 14, 17-19, 22-24, 26, 28, 30, 32-34, 38]. However generally flow cytometric studies with formalin-fixed and paraffin-embedded material showed a low rate of DNA-non diploid tumors. In the reported studies it ranged between 42% and 93%. In our study 66% of cervical carcinomas were DNA-heterogeneous, so the DNA index obtained from a single sample might not be

representative of the entire tumor. Also, the choice of treatment interfaces with the survival of patients with aneuploid tumors since DNA aneuploid tumors may be more sensitive to radiation therapy [11, 25, 37]. The present study was prospective, all patients had the same primary therapy and the DNA measurements were based on multiple samples on fresh tumor with an internal DNA reference and with external histological control of the measured tissue.

Our study also analyzed clinical and histological variables. Like other authors we found FIGO stage, histological parametrial involvement, age, grade and distant metastases to be prognostic factors [36]. FIGO stage seems to be the major prognostic factor available before treatment [12]. Parametrial involvement and distant metastases have long been recognized as unfavorable prognostic factors. The importance of histopathologic grading for disease-free survival (DFS) depends on the grading system which is applied. Generally, morphologic grading of malignancy is subjective and poorly reproducible. In our series G1 carcinomas were associated with better DFS (0.83) than G2 (0.62) or G3 carcinomas (0.67). Furthermore patients between 40 - < 50 years of age had a tendency toward better DFS (0.85) than patients < 40 years (0.65) and patients ≥ 50 years (0.48). This was not statistically significant and in the literature the influence of the age of the patients on relapse and survival is controversial [36].

In conclusion, this study indicates that DNA ploidy and DNA heterogeneity are prognostic factors in cervical cancer. DNA flow cytometry may be used preoperatively to identify low-risk and high-risk patients within a given FIGO stage.

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