Prognostic Value of Genomic Alterations in Invasive Cervical Squamous Cell Carcinoma of Clinical Stage IB Detected by Comparative Genomic Hybridization[1]

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ABSTRACT

The clinical behavior of invasive cervical carcinoma of clinical stage IB varies considerably in tumors presenting without regional lymph node metastases. The early identification of patients at higher risk for poor outcome may prove useful because these patients would benefit from aggressive adjuvant treatments. In this study, comparative genomic hybridization was applied to evaluate whether genomic aberrations have prognostic significance in cervical carcinoma. Genomic alterations were evaluated in 62 cervical carcinomas of clinical stage IB. DNA sequence losses were most prevalent at chromosomes 4q (53%), 3p (52%), 13q (45%), 4p (44%), Xq (44%), 5q (40%), 18q (37%), and 6q (35%). Several genomic alterations were associated with poor clinical outcome or metastasis. The total number of DNA aberrations/tumor ($P < 0.02$) and the number of DNA sequence losses/tumor ($P < 0.04$) were associated with disease-specific survival. 9p deletions were significantly more frequent in carcinomas with lymph node metastasis than in node-negative tumors ($P < 0.03$). Losses of chromosome 11p ($P < 0.0001$) and 18q ($P < 0.01$) were associated with poor prognosis in cervical carcinomas without lymph node metastasis. These data suggest that inactivation of tumor suppressor genes on chromosomes 9p, 11p, and 18q may play a role in the progression of cervical carcinoma.

INTRODUCTION

Worldwide, cervical carcinoma is the second most common malignancy in women in both incidence and mortality (1). Despite substantial advances in the understanding of the molecular events involved in the development of many epithelial cancers, such as colon, breast, and lung cancers, the molecular pathogenesis of cervical cancer is less well known. Oncogenic types of HPVs[3] have been found in more than 95% of cervical carcinomas, but only a small fraction of women harboring oncogenic HPV in their lower genital tract will develop cervical cancer. Thus, HPV infection alone appears to be insufficient for the progression to a malignant phenotype and suggests the involvement of other genetic and/or epigenetic events in cervical carcinogenesis (2, 3). Frequent loss of heterozygosity has been demonstrated for chromosomes 3p, 4p, 4q, 9p, and 11q (4), suggesting that alterations of putative suppressor genes on these chromosomes may contribute to tumor progression.

It has recently been suggested that gains of chromosome 3q sequences could be the most consistent chromosomal aberration during the transition from high-grade cervical intraepithelial neoplasia to early invasive cervical carcinoma (5). Gains on chromosomes 1q, 3q,

and 5p (6) were shown to be particularly frequent in advanced-stage cervical carcinomas (clinical stage IIB–IV).

Thus far, no data exist about the prognostic significance of chromosomal aberrations in primarily surgically treated invasive cervical carcinomas of clinical stage IB. Genetic changes with prognostic relevance might lead to improved methods of predicting prognosis and thus may help in treatment planning. In this study, CGH was applied to evaluate the prognostic significance of DNA aberrations in radically operated squamous cell carcinomas confined to the cervix (International Federation of Gynecology and Obstetrics, clinical stage IB). CGH allows a survey of all DNA copy number changes of a given tumor (7).

MATERIALS AND METHODS

Tumors. Tissue probes of 62 radically operated invasive squamous cell carcinomas of clinical stage IB were fixed in 4% buffered formalin, embedded in paraffin, and routinely stained for histological diagnosis. Tumor stage and grade were defined according to International Union Against Cancer and WHO classifications (8). Forty-three women with invasive carcinomas had no regional lymph node metastases at the time of operation, whereas lymph node metastases were histologically confirmed in 19 patients. Clinical follow-up data were available for all patients. Disease-specific survival was defined as the time between primary treatment and death of patients due to the tumor. All samples of invasive cervical carcinoma were positive for high-risk HPV DNA of HPV types 16/18 (9). Pathomorphological parameters (depth of invasion, vascular invasion, histological grade, microvessel density, and peritumoral inflammation) were analyzed by one pathologist (J. T.). Nuclear proteins (p27^{Kip1}, c-myc, p53, Ki-67, and cyclin E) have been detected by immunohistochemistry as published previously (10, 11).

Tissue Preparation. Specimens were trimmed to enrich for tumor by excising tumor tissue from the paraffin block. The excised tumor tissue was re-embedded in a paraffin block. Tissue sections (5 μm) were cut from these tumor blocks. The first and the last sections were stained with H&E to ensure a minimum of 75% tumor cells in the sample. Normal tissue was scraped away using a scalpel, if necessary.

DNA Preparation. DNA extraction and labeling were performed as described previously (12). The 20-μm-thick sections were deparaffinized and suspended in DNA extraction buffer containing 0.5 mg/ml proteinase K. Additional proteinase K was added 24 and 48 h later for a total incubation time of 72 h. Tumor DNA (2 μg) was nick-translated by using a commercial kit (BioNick kit; Life Technologies, Inc., Gaithersburg, MD); SpectrumGreen Direct-labeled Total Human Genomic dUTPs (Vysis, Inc., Downers Grove, IL) was used for direct labeling of tumor DNA. SpectrumRed-labeled normal reference DNA (Vysis, Inc.) was used for cohybridization.

CGH and Digital Image Analysis. CGH and digital image analysis were carried out as described previously (13). The hybridization mixture consisted of 200 ng of SpectrumGreen-labeled tumor DNA, 200 ng of SpectrumRed-labeled normal reference DNA, and 20 μg of human Cot-1 DNA (Life Technologies, Inc.) dissolved in 10 μl of hybridization buffer [50% formamide, 10% dextran sulfate, and 2× SSC (pH 7.0)]. Hybridization was performed over 3 days at 37°C to normal metaphase spreads (Vysis, Inc.). Posthybridization washes were performed as described previously (14). Digital images were collected from six to seven metaphases using a Photometrics cooled charge coupled device camera (Microimager 1400; Xillix Technologies, Van-

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couver, British Columbia, Canada) and a Sun workstation. The Vysis software program was used to calculate average green:red ratio profiles for each chromosome. At least four observations/autosome and at least two observations/sex chromosome were included in each analysis according to previous recommendations by Kallioniemi *et al.* (15).

Controls and Threshold Definitions. CGH experiments included a tumor cell line (Spectrum Green-labeled MPE-600 DNA; Vysis) with known aberrations (positive control) and a hybridization of two differentially labeled sex-mismatched normal DNAs to each other (negative control). Sex-mismatched normal controls were also used to test the ability of each metaphase batch to allow for a linear relationship between fluorescence intensities and DNA sequence copy numbers. Metaphases were used only if the color ratio of sex-mismatched normal DNAs was ≤0.66 at the X chromosome. The thresholds used for definition of DNA sequence copy number gains and losses were based on the results of CGH analyses of formalin-fixed normal cervical tissues. Gains of DNA sequences were defined as chromosomal regions in which both the mean green:red fluorescence ratio and its SD were >1.20, whereas losses were defined as regions in which both the mean and its SD were <0.80. Overrepresentations were considered amplifications when the fluorescence ratio values in a subregion of a chromosome arm exceeded 1.5. In negative control hybridizations, the mean green:red ratio occasionally exceeded the fixed 1.2 cutoff level at the following chromosomal regions: 1p32-pter; 16p; 19; and 22. These known GC-rich regions were therefore excluded from all analyses.

FISH Analysis. To further evaluate the prevalence of 3q gains in cervical carcinoma by FISH, a cervical carcinoma tumor microarray was constructed as described previously (16). Briefly, core tissue biopsies (diameter, 0.6 mm) were taken from all individual paraffin-embedded cervical carcinomas (donor blocks) and precisely arrayed into a new recipient paraffin block (35 × 20 mm) with a custom-built precision instrument (Beecher Instruments, Silver Spring, MD). Sections (5 μm) were cut for FISH analysis. The presence of tumor tissue on the arrayed samples was verified on one H&E-stained section.

Two-color FISH of sections of the arrayed formalin-fixed samples was performed using a Spectrum Orange-labeled 3q probe (TERC, hTR; 3q26) with a corresponding FITC-labeled centromeric 3 α satellite probe (Vysis, Inc.). Slide pretreatment, hybridization, and washing procedures were performed as described previously (17). The hybridization mixture contained 3 μl of each of the probes and Cot-1 DNA (1 mg/ml). Slides were counterstained with 0.2 μM 4′,6-diamidino-2-phenylindole. FISH signals were scored with a Zeiss fluorescence microscope equipped with a double-band pass filter using ×40–×100 objectives.

To avoid misinterpretation due to inefficient hybridization, cells were only counted if at least one bright centromere signal and one signal of the 3q probe were present. The relative number of gene signals in relation to centromeric signals was evaluated. A gain of chromosome 3q was defined as the presence of more 3q than centromere 3 signals in more than 10% of nuclei.

Statistics. The χ^2 test was used to evaluate relationships between categories. Disease-specific survival was defined as the time between primary treatment and death due to the tumor. Patients alive at the time of last follow-up were censored. Survival analysis was done by the Kaplan-Meier method with a log-rank test. The medians (total number of DNA aberrations and DNA sequence losses and gains) were used as cutoff points to define patients with high and low numbers of aberrations. The Cox proportional hazard model with stepwise selection of variables was applied to test for independent prognostic significance. Statistical analyses were performed using the StatView 4.5 Software program (Abacus Concepts, 1995).

RESULTS

Overview of Genetic Changes in Cervical Carcinoma. Fifty-six of the 62 cervical carcinomas of clinical stage IB showed either relative DNA sequence losses (56 of 62 carcinomas) or gains (43 of 62 carcinomas) by CGH. There was a median of 5 total aberrations/tumor (range, 0–18 aberrations/tumor), 4 losses (range, 0–13 losses), and 1 gain (range, 0–5 gains). Losses were most frequently observed on chromosomes 4q (53%), 3p (52%), 13q (45%), 4p (44%), Xq (44%), 5q (40%), 18q (37%), and 6q (35%). Gains frequently involved chromosome 17p (30%), 17q (27%), 20q (16%), and 3q (15%). No high-level DNA amplifications were detected. Only losses

Table 1 *Specific genomic alterations and regional lymph node status*

Type of aberration	Locus	No. of patients without regional LNM ($n = 43$)[a]	No. of patients with regional LNM ($n = 19$)	P[b]
Deletion	2q	14	5	0.62
	3p	21	11	0.51
	4p	19	8	0.88
	4q	25	8	0.24
	5q	18	7	0.71
	6q	16	6	0.67
	9p	11	10	**0.03**
	11p	9	3	0.64
	11q	8	4	0.82
	13q	20	8	0.75
	18q	18	5	0.24
	Xp	10	6	0.63
	Xq	18	9	0.56
Gain	3q	7	2	0.55
	9q	4	3	0.46
	17p	15	4	0.27
	17q	13	4	0.45
	20q	7	3	0.96

[a] LNM, lymph node metastasis.
[b] χ^2 test.

of 9p were significantly more frequent in patients with regional lymph node metastasis ($P = 0.03$) than in node-negative patients (Table 1).

There was no difference in the number of aberrations, DNA sequence losses, and gains between patients with and without regional lymph node metastasis.

Clinical Outcome. Survival data were available for all patients, with a mean follow-up of 76.4 ± 59.5 months (median, 64 months). Clinical outcome of patients with regional lymph node metastasis was significantly worse than that of node-negative patients ($P < 0.001$). Therefore, survival analysis to study the association of genetic changes with clinical outcome was restricted to patients without regional lymph node metastasis, whose mean follow-up time was 87.4 ± 57.3 months (median, 71 months).

Genetic Changes and Clinical Outcome in Cervical Carcinoma without Regional Lymph Node Metastasis. The most prevalent DNA sequence losses in 43 patients without regional lymph node metastasis occurred on chromosomes 4q (58%), 3p (49%), 13q (47%), 4p (44%), 5q (42%), 18q (42%), and Xq (42%). Frequent DNA sequence gains were found on chromosomes 17p (35%) and 17q (30%). Copy number aberrations for patients without regional lymph node metastasis are shown for the entire genome in Fig. 1. A high total number of genetic aberrations (Fig. 2*A*; $P < 0.02$) and a high number of DNA sequence losses (Fig. 2*B*; $P < 0.04$) were strongly associated with short disease-free survival. The number of gains was not associated with patient prognosis.

The most common individual loci were tested to see which were most strongly linked with survival. Losses in chromosomes 11p and 18q were the only individual loci associated with clinical outcome. The disease-free survival of patients with 11p (Fig. 2*C*; $P < 0.0001$) and 18q (Fig. 2*D*; $P < 0.01$) deletions was clearly worse compared to patients without 11p and 18q deletions. None of the other aberrations tested, including DNA sequence losses on 4q, 3p, 13q, 4p, 5q, and Xp and gains on chromosome 17, were associated with prognosis.

Multiparameter Analysis. Cox stepwise regression analysis of pathomorphological parameters (depth of invasion, histological grade, vascular invasion, microvessel density, and peritumoral inflammation) and genomic DNA aberrations (total number of DNA losses and loss of 11p and 18q) revealed DNA sequence losses of chromosome 11p to be of independent prognostic significance ($P < 0.001$). In a separate, previous analysis (10, 11), immunohistochemically analyzed nuclear proteins in cervical neoplasia (p27[Kip1], c-myc, p53, Ki-67 labeling index, and cyclin E) and genomic DNA aberrations were
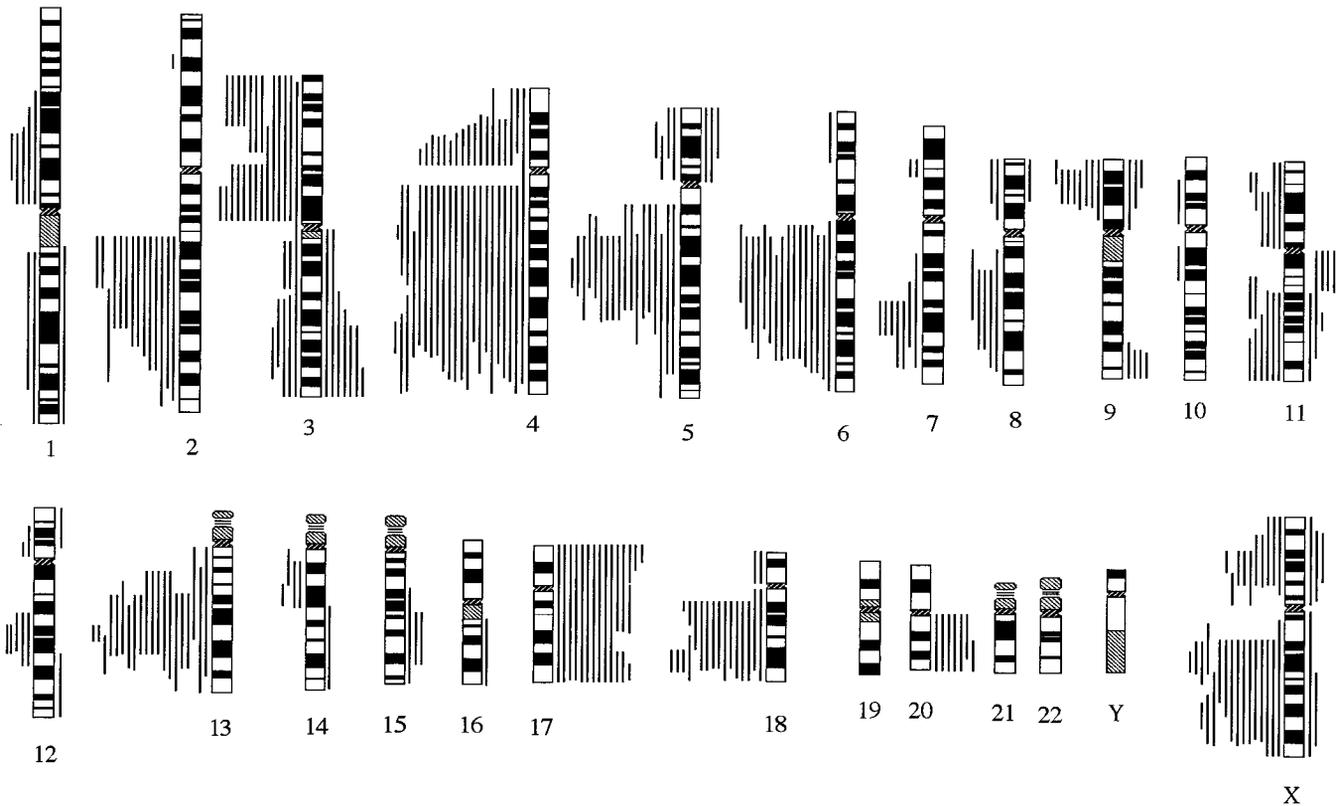
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Fig. 1. Summary of all gains (*right*) and losses (*left*) of DNA sequences observed by CGH in 43 invasive cervical squamous cell carcinomas without regional lymph node metastasis.

tested for independent prognostic significance. Within this group, Cox regression analysis demonstrated the total number of DNA sequence losses/tumor ($P < 0.0001$) and losses on chromosome 18q to be of prognostic importance ($P < 0.005$).

FISH Analysis. After optimizing pretreatment of tumor array sections, high-quality hybridization signals for both centromeric and gene-specific probes were obtained in 34 tumors. Failure of FISH was mostly due to decreased hybridization efficiency in some tumors. All tumors with unsuccessful hybridization were excluded from analysis. Low-level copy number gains with more 3q than centromere 3 signals in more than 10% of cells were detected in 19 of 34 tumors (56%). Most of the tumors contained three or four signals for the 3q26 locus and two signals for the centromere 3 probe. There were no high-level 3q amplifications found in the 34 evaluable tumor samples.

DISCUSSION

The major aim of this study was to evaluate the prognostic significance of the number of genetic aberrations for patients with invasive carcinoma of clinical stage IB. In patients without regional lymph node metastasis, our study showed an association between the total number of aberrations and overall survival. The detailed analysis showed that DNA sequence losses and not gains were responsible for this result. This observation is analogous to results obtained in studies of breast (18) and kidney cancer (12) in which a significantly worse prognosis was seen in tumors with a high number of DNA sequence losses compared to tumors with a low number of losses. Because chromosomal deletions may inactivate tumor suppressor genes, our finding supports the hypothesis that inactivation of multiple suppressor genes underlies cervical cancer progression (19, 20).

In this study, DNA losses of chromosomes 18q and 11p were associated with poor prognosis. Three candidate tumor suppressor genes including *DCC* (21), *DPC4* (22), and *MADR2* (23) have been identified on the long arm of chromosome 18. Two of these genes, *DPC4* and *MADR2,* are particularly interesting because they are important mediators in the transforming growth factor β pathway. For the *DCC* gene, Klingelhutz *et al.* (24) showed that progression of HPV-transformed keratinocytes to tumorigenic cells was accompanied by a loss of heterozygosity affecting the *DCC* gene, resulting in loss of DCC expression. Tumorigenicity was suppressed when a DCC expression vector was transfected back into these cells, thus concluding that the *DCC* gene suppresses the malignant phenotype of transformed epithelial cells (25). Loss of 18q might therefore contribute to a more aggressive tumor cell phenotype in cervical cancer and thus to an unfavorable disease outcome.

Chromosome 11 is the second most frequent chromosome that is structurally or numerically aberrant in all neoplasias (26). The significant difference in disease-specific survival between tumors with and without 11p losses suggests that a tumor suppressor gene on 11p is involved in cervical carcinoma progression. Of particular relevance to the interaction of cellular genes with those of HPVs in cervical carcinoma (27) is the evidence that 11p is likely to contain a gene or genes involved in the regulation of the HPV-16 early enhancer promoter and in the suppression of the transforming activity of the viral DNA (28).

There are few data available relating specific genetic events to metastatic behavior in cervical cancer. Interestingly, an association of 9p deletion with the presence of regional lymph node metastases was found in this study. This is consistent with previous findings in renal cancer and other solid tumors in which a relationship between 9p deletion and development of metastasis has also been described (12, 29, 30). Whether abnormalities and deletions of putative tumor suppressor genes on chromosome 9p may be a significant event in the
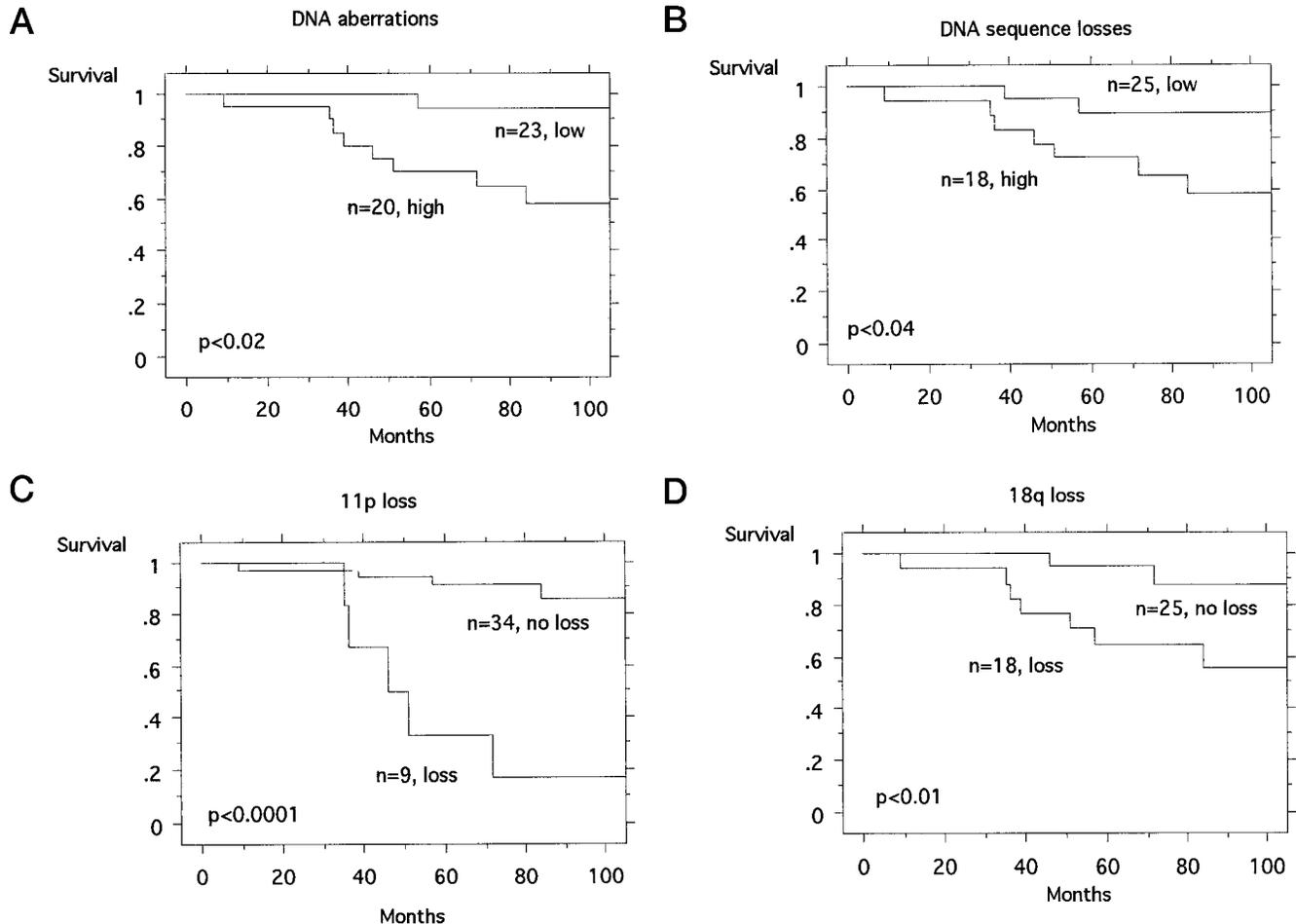
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Fig. 2. Survival in patients without regional lymph node metastasis ($n = 43$). *A*, patients with low (≤ 5 DNA aberrations/tumor) and high (>5 DNA aberrations/tumor) numbers of DNA aberrations. *B*, patients with low (<5 DNA sequence losses/tumor) and high (≥ 5 DNA sequence losses/tumor) numbers of DNA sequence losses. *C*, patients with and without loss of chromosome 11p. *D*, patients with and without loss of chromosome 18q.

progression of cervical carcinoma remain to be determined in additional investigations.

Other frequent aberrations in cervical carcinoma not related to metastasis or prognosis included losses of chromosomes 4q, 3p, 13q, and 4p and gains of chromosomes 17p and 17q. Interestingly, a high prevalence of 4q and 4p losses has been described by Larson *et al.* (19) in high-grade cervical intraepithelial neoplasia. This argues for a decisive role of a disturbed function of one or several genes on chromosome 4 in the early stages of cervical neoplasia (19).

Chromosome 13q loss has also been found by microsatellite analysis and indicates various pathways for the inactivation of the retinoblastoma suppressor gene (*RB1*). There is evidence that high-risk oncogenic HPV types inactivate the RB1 gene product by the viral E7 oncogene product in cervical carcinomas (31). The prevalence of 13q losses indicates that an additional mutational loss of the *RB1* gene might be involved in many cervical carcinomas.

DNA sequence losses involving chromosome 3p were the second most frequent aberration (49%) in our study. Our results are consistent with recently reported data describing progressive deletions at one or more regions at 3p as frequent and early events in the pathogenesis of cervical carcinoma (4). These findings suggest a pivotal role for 3p deletions in the pathogenesis and progression of cervical carcinomas.

DNA gains most often involved chromosome 17p (30%) and 17q (27%). Previous studies have detected amplification (32) or overexpression (33) of the *HER-2/neu* gene on chromosome 17q21–22 in 14–22% of cervical carcinomas. However, these studies have also

shown that overexpression of the *HER-2/neu* gene plays a minor role in the oncogenesis of cervical cancer.

In this study, 3q gains were detected in a subset of cervical carcinoma by CGH. These data are in contrast to the findings of Heselmeyer *et al.* (5). They detected 3q gains in only 1 of 13 severe dysplasias but in 9 of 10 invasive pT1 carcinomas (90%), suggesting that almost all invasive cervical carcinomas are characterized by 3q gains. In advanced cervical carcinomas, they detected 3q gains in 23 of 30 tumors. The discrepancy between the CGH data of Heselmeyer *et al.* (5) and our data is relevant because 3q gains could be used as a possible molecular marker for cervical cancer detection. The reasons for the variations in the prevalence of 3q gains is unclear. Both studies used formalin-fixed tissue for DNA extraction and CGH analysis. Also, similar thresholds were defined for interpretation of the ratio profiles. There were no evident racial or ethnical differences in the patient set, and both studies included HPV-positive pT1 carcinomas. In our study, many tumors showed other clear aberrations, but not 3q gains, suggesting that methodological shortcomings might not be responsible for this discrepancy.

Because of the discrepancy between these CGH studies, we performed a FISH analysis using our recently developed tumor microarray technology (16). This analysis confirmed that 3q gains are not present in all cervical carcinomas. It was not surprising that FISH detected a higher prevalence of 3q gains compared to CGH (56% *versus* 15% of all carcinomas) because CGH is primarily a screening method that will not find all low-level gains. The FISH analysis

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clearly showed that most tumors contained low-level gains with only one or two more 3q26 copies than centromere 3 copies. Such low copy number gains can be missed by CGH due to admixture with nontumorous cells (lymphocytes and endothelial cells).

In summary, our data suggest that the total number of DNA aberrations and the total number of DNA sequence losses may be of prognostic significance in patients with invasive cervical cancer of clinical stage IB without regional lymph node metastasis. In addition, these results highlight several chromosomal regions that may harbor important genes for cervical carcinoma. Losses of DNA sequences on chromosomes 11p and 18q may be relevant for progression in invasive cervical carcinoma. Losses of DNA sequences of 9p may be relevant for cervical carcinoma metastasis. This is the first study reporting an association between a high number of genomic alterations as well as a high number of DNA sequence losses with worse prognosis in cervical cancer of clinical stage IB. Additional studies will not only have to identify the target genes of these alterations but will also have to evaluate whether the detection of these cytogenetic changes can provide clinically useful information.

ACKNOWLEDGMENTS

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