Genomic Changes and HPV Type in Cervical Carcinoma (44497)

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Abstract. **To identify chromosomal regions that may include the loci of abnormally expressed cellular genes and may be specifically altered depending on the histological subtype of the tumor, we studied primary cervical carcinoma using CGH and HPV genotyping. Eighty-seven percent of the primary tumors were positive for DNA of a "high-risk" HPV type (e.g., 16 or 18). In the cervical carcinomas, without reference to histologic subtype, overrepresentation of chromosome 3q was the most consistent chromosomal aberration with underrepresentation of chromosome 3p also a frequent finding. Chromosome arms 1q, 5p, 20q, and Xq were overrepresented in many tumors and 3p loss and 5p, 8q, and 16q gain were only associated with squamous cell carcinoma in this series.** [P.S.E.B.M. 2000, Vol 223]

Approximately 500,000 new cases of cervical carcinoma are diagnosed worldwide each year. With a worldwide survival rate of only 40%, cervical carcinoma remains a key international health problem.

Development of cervical carcinoma is a multistage process. Generally, cervical carcinoma arises from histopathologically defined precursor lesions referred to as high-grade intraepithelial lesions (HSIL). These precursor lesions may arise from either human papillomavirus (HPV)-induced low-grade lesions (LSIL), or directly from HPV-infected histopathologically normal epithelium (1). This disease is strongly associated with HPV infection, with up to 95% of cervical squamous cell carcinomas containing detectable HPV DNA sequences (2). To date, more than 80 HPV types have been identified and approximately 30 of these have been shown to be associated with lesions of the anogenital tract. This subset of HPV types can be further subdivided into "low-risk" (e.g., HPV 6, 11), associated with LSIL; "intermediate-risk" (e.g., HPV 33, 35); and "high-risk" (e.g., HPV 16, 18), associated with malignant transformation. The ability of "high-risk" HPVs to contribute to malignant progression is a consequence of continued expression of the viral *E6* and *E7* oncogenes (3). The major effect of *E6/E7* expression is to abrogate cell cycle controls and initiate a process through which host cell genomic changes accumulate and lead to a malignant phenotype. In an earlier cytogenetic analysis of a series of HPV-immortalized epithelial cell lines, structural rearrangements were most common in chromosomes 1, 3, and 5, with less frequent aberrations in chromosomes 7, 8, 12, 13, 16, and 22 (4).

Previous studies of loss of heterozygosity (LOH) in cervical carcinoma have shown 3p, 6p, and 18q to be the most commonly deleted chromosome regions in the genome (5). At present there is a limited amount of cytogenetic information available from direct preparations or from short-term cultures of cervical carcinomas (6, 7). Published studies have shown cervical tumors to have a variable modal chromosome number ranging from 40 to 100, with complex karyotypes including several unidentifiable markers. Chromosomes 1, 3, 11, and 17 have been reported to be involved most frequently in structural aberrations, and a small metacentric, i(4p) or i(5p), was described as the most frequent single anomaly (7). Conventional G-banding of complex karyotypes, such as these, nearly always results in a number of unidentifiable or misidentified regions and markers. We used the technique of comparative genomic hybridization (CGH) to avoid the problem of misidentification of chromosomes and to allow screening of the entire tumor genome for gains and losses in a single experiment. CGH makes it possible to detect chromosomal changes in tumors without specific probes. This method is based on

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cohybridization of differentially labeled total genomic tumor DNA and normal DNA to normal metaphase chromosomes (8). Previous CGH studies of cervical tumor progression have shown that chromosome 3q gain defines the transition from preinvasive to invasive cervical tumors (9). There have been few studies comparing the chromosomal aberrations that occur in the different histologic subtypes of cervical carcinoma. Most studies have focused on squamous cell carcinoma, which is the most commonly occurring subtype (≈ 75% of cases), with adenocarcinoma making up most of the remainder. A recent study of adenocarcinoma of the cervix indicated that patterns of allelic loss seem to differ between adenocarcinoma and squamous cell carcinoma. For example, Ferguson *et al.* (10) did not identify any adenocarcinomas with LOH of markers on 3p, a very common site of LOH in squamous cell carcinomas.

Materials and Methods

Cells and Tissue Culture. Primary human foreskin keratinocytes (HFKs) were cultured from neonatal foreskins. Primary human cervical keratinocytes (HCKs) were cultured from histologically normal adult cervical tissue. The 1811 cell line was previously established by cotransfection of HFKs with HPV type 18 DNA and pSV2neo (4). The 1811-NMU-T1 cell line is a tumorigenic cell line generated by NMU treatment of early passage 1811 cells (11). All cells were grown in keratinocyte serum–free medium (KSFM) (Gibco BRL) as previously described.

Tumor Material. A total of 27 snap-frozen tissue specimens of cervical carcinomas comprised the material for this study. Tumor specimens were obtained after appropriate consent was granted from patients who were treated at the University of Washington Medical Center, Seattle, Washington. Histologic diagnosis was performed at the University of Washington Medical Center. Of the 27 carcinomas, 15 were squamous cell carcinomas, 9 were adenocarcinomas, 2 were adenosquamous carcinomas, and 1 was a small-cell carcinoma with neuroendocrine differentiation.

DNA Preparation. *1811 and 1811-NMU-T1 studies.* As reference DNA for the cell line studies, genomic DNA was isolated from HFK cells. As test DNA, genomic DNA was isolated from early-, mid-, and late-passage 1811 cells as well as early-passage 1811-NMU-T1 cells.

Cervical carcinoma studies. As reference DNA for the cervical carcinoma CGH experiments, genomic DNA was prepared from HCK cells. DNA extraction from all cell lines was done following standard protocols (12). For tumor DNA extraction, snap-frozen tumor samples were extracted following standard procedures (12). DNA concentration was measured using a Beckman Du-64 spectrophotometer.

HPV Detection and Typing. The polymerase chain reaction (PCR)-based assay for HPV detection was carried out as described previously (13). Briefly, PCR was performed using L1 consensus primers (14) and also primers specific for the E6 open reading frames (ORFs) of HPV 16 and 18. The identity of the PCR products was confirmed by Southern hybridization, and the L1 consensus products typed by restriction fragment analysis (15).

TRAP Assay. The TRAP assay was performed as described previously. Briefly, 0.1 μg of CX primer ([5′-$(CCCTTA)_3CCCTAA$-3′]) was lyophilyzed in a 0.6 ml Eppendorf tube and then sealed below a bead of AmpliWax (PE Biosystems, Foster City, CA). The telomerase reaction above the wax involved a [γ-^{32}P] ATP labeled TS primer (5′-AATCCGTCGAGCAGAGTT-3′). 0.1 μg of TS primer was used in each reaction at 25×10^6 counts/min/μg of primer. The reaction mix was made as previously described. Either 2.5 μg, 0.25 μg, or 0.025 μg of protein were used for each reaction. The reaction was incubated for 30 min at room temperature. During this time, the TS primer was used by any telomerase present for the addition of TTAGGG repeats. The sample was then subjected to 3 min at 90°C to melt the wax barrier and 27 cycles of polymerase chain reaction (PCR) as described previously. One tenth of the reaction was run on an 8% nondenaturing polyacrylamide gel, after which the gel was dried and exposed to film for 24 hr at –80°C.

Telomere Length Measurement. Genomic DNA (3 μg) was digested with the enzyme Bgl II (Gibco/BRL, Rockville, MD) according to manufacturer's protocol, run on 0.7% agarose gels, and blotted onto Hybond N^+ membranes (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) as previously described (16). Telomeric TTAAGGG repeat probe was ^{32}PCTP random primed by using a random priming kit (Roche Diagnostics Corporation/Roche Molecular Biochemicals, Indianapolis, IN). Blots were hybridized and washed as described previously (16). Hybridized blots were exposed to XAR-5 film (Eastman Kodak Co., Rochester, NY).

Chromosome Preparations. Phytohemagglutinin-stimulated lymphocytes from normal female subjects (46, XX) and normal male subjects (46, XY) were cultured in AIMV medium (Gibco BRL). Mitotic cells were arrested with colcemid, treated with a hypotonic solution of 0.068 *M* KCL, and fixed in methanol/acetic acid (3:1). Metaphase spreads were made following standard procedures.

Labeling of DNA Probes for Comparative Genomic Hybridization (CGH). Test DNA was labeled with biotin-14-dATP (Gibco BRL) and reference DNA with digoxigenin-11-dUTP (Boehringer Mannheim) using the standard protocol provided with the Boehringer Mannheim nick translation kit. CGH results were confirmed by reversing the labeling of the test and reference DNAs.

CGH Analysis. CGH was performed as described previously (17) with minor modifications. Normal metaphase spreads were pretreated with pepsin (50 μg/ml), 0.01 *M* HCl for 10 min at 37°C, followed by postfixation with 1% formaldehyde in phosphate-buffered saline (1× PBS + 50 m*M* $MgCl_2$) for 10 min at RT. The slides were then denatured in 70% formamide, 2× SSC at 70°C for 2–3 min, and dehydrated in a series of ice-cold ethanol. Then 1 μg

each of labeled tumor DNA and labeled reference DNA and 80 μg of unlabeled human Cot-1 DNA fraction (Gibco BRL) were dissolved in 12 μl hybridization solution (50% formamide and 10% dextran sulfate in 2× SSC). This probe mixture was denatured for 5 min at 80°C and allowed to preanneal for 1–2 hr at 37°C. It was then placed on the denatured slides, coverslipped, and sealed with rubber cement. The slides were then hybridized for 3–5 days at 37°C.

For probe detection, slides were washed three times in 50% formamide, 2× SSC at 45°C for 5 min each, followed by 3× 5-min washes in 0.1× SSC at 60°C. Slides were preincubated in 3% BSA, 4× SSC/0.1% Tween 20 at 37°C for 30 min. Biotinylated DNA sequences were detected using avidin-fluorescein isothiocyanate (FITC) (Vector Laboratories, Burlingame, CA). Signals were amplified once according to Pinkel *et al.* (18). Digoxigenin-labeled DNA sequences were visualized using rhodamine-conjugated sheep antidigoxigenin Fab fragments (Boehringer Mannheim). The preparations were then counterstained with 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI)(50 ng/ml) for 10 min at RT and mounted in vectashield (Vector labs).

Image Acquisition and Processing. Image acquisition and processing were performed using an epifluorescence microscope (Nikon Microphot-SA, Melville, NY) equipped with a 100w mercury lamp and a standard CCD camera interfaced to a DELL PC. Gray level images were recorded separately for each fluorochrome using specifically aligned filter sets for DAPI, FITC, and rhodamine. Digital images were processed with Cytovision software developed by Applied Imaging. After correction of centromere placement and determination of the chromosome axis, individual FITC/rhodamine profiles were calculated for each chromosome. Mean ratio profiles were then determined from 9 to 25 metaphase spreads. Chromosomes were identified by inspection of inverted digital DAPI images.

Results

In this study, we used CGH to identify chromosomal aberrations occurring in cervical carcinoma and then compared the chromosomal aberrations detected based on histologic subtype and HPV status of the tumor. A total of 27 snap-frozen tissue specimens of cervical carcinomas comprised the material for this study. Of the 27 carcinomas, 15 were squamous cell carcinomas, 9 were adenocarcinomas, 2 were adenosquamous carcinomas, and 1 was a small-cell carcinoma with neuroendocrine differentiation.

To initiate the studies an HPV-18 immortalized epithelial cell line was analyzed by CGH to identify chromosomal aberrations that occurred as the cells progressed from an immortal to a tumorigenic phenotype after carcinogen treatment. The results were compared to previously reported cytogenetic findings for this cell line and were consistent with earlier findings (19).

A summary of chromosome aberrations detected with CGH in a series of primary uterine cervical carcinomas is shown in Figure 1. Twenty-three tumors showed gains, and 16 (of 27) tumors showed losses at one or more chromosomal regions. Three tumors contained no detectable changes. On average, there were 4.67 aberrations per tumor (range 0–10). Figure 2 shows representative normal and tumor CGH profiles. Gains most often recorded were on chromosomes 1, 3, 20, and X. Increased copies of 3q were detected in 15 tumors, with all gains including the region 3q24-q26.1. Gain of Xq usually involved the entire chromosome arm; however, one case showed a smaller amplification encompassing Xq22-qter. Gain of 20q occurred in seven tumors with six of those tumors gaining the entire chromosome arm and one gaining only 20q13.2-qter. Several other chromosomal regions, such as 5p (minimal common region 5p12-p15.2), 8q, 9p, 15q, and 16q were also gained. Decreased copy number was most often detected at 3p with the minimal common region involved in DNA loss at 3p13-p14. Several other chromosomal regions showed less frequent loss (Table I).

The most common aberrations detected by CGH were reexamined based on HPV status and histologic subtype of the tumors. Overall, 24 of the tumors were positive for "high-risk" HPV DNA. Of those HPV-positive tumors, 11 were positive for HPV 16, 10 for HPV 18, and 3 for other "high" or "intermediate-risk" HPVs, including HPV 35, 45, and 73. There was no correlation between HPV type and chromosomal aberration detected in the tumors. When chromosomal aberrations detected and histologic subtype of the tumors were compared, it became apparent that all tumors showing loss of 3p and at a lower frequency 6q loss were

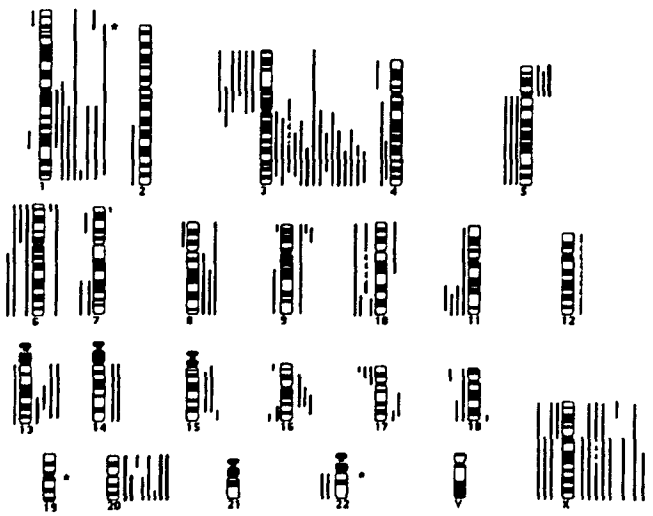

Figure 1. Summary of gains and losses of DNA sequences in 27 primary uterine cervical carcinomas detected by CGH. Vertical lines on the left-hand side of the chromosome diagrams correspond to loss of genetic material in the tumor, and those on the right side correspond to gains. Bold lines on the right side correspond to amplifications (green to red ratio ≥ 1.5). Each line illustrates the region of the chromosome affected in one single tumor. The band locations of the genetic changes were estimated based on DAPI banding. *Fixed decision limits for the loss (green to red ratio ≤ 0.75) and gain (≥ 1.25) of DNA sequences were used for all other chromosomal sites.

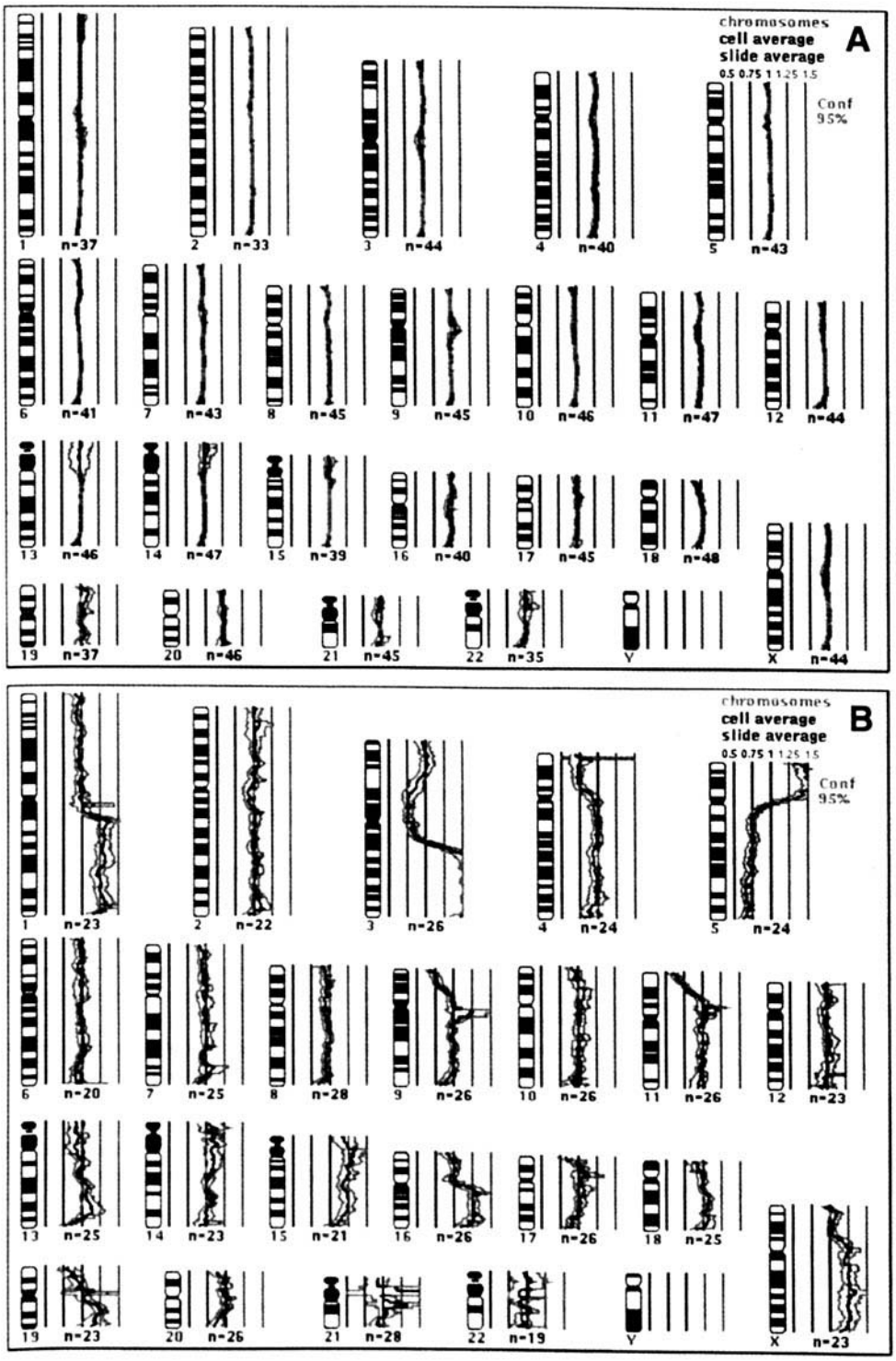

Figure 2. Mean green to red ratio profiles from pter to qter, obtained from CGH analysis of two primary uterine cervical carcinomas. Panel A is an example of a normal CGH profile from one sample. The profile shows no evidence of DNA sequence copy number changes. The small increase in the ratios at the heterochromatic regions throughout the genome (for example the heterochromatic region of chromosome 9) is excluded from analysis as it represents repeated sequences that are blocked by the use of unlabeled Cot-1 DNA in the hybridization. Panel B is an example of a profile of one of the cervical tumors exhibiting chromosomal changes. Note the aberrations of chromosomes 1, 3, 4, 5, 11, and X.

SCCs. Gain of 5p, 8q, and 16q was also restricted to SCC diagnosis (Table I).

Discussion

Telomerase activity can be detected in the majority of cervical tumors (13) and has been shown to be induced by expression of the HPV-16 E6 gene (20). Recently, the human telomerase catalytic subunit (hTERT/hEST2) has been cloned (21) and mapped to chromosome 5p15.33 (22). Takakura *et al.* (23) have reported a strong correlation of telomerase activity with hTERT mRNA expression. Increased transcription of a gene can be achieved by amplifying the locus containing the gene; therefore, we compared the level of telomerase activity by TRAP assay (24), seen in tumors with or without gain of 5p, to determine if there was a quantitative difference in telomerase activity that correlated with gain of chromosome 5p material. The results

Table I. Comparison of Histological Subtype, HPV Type and Chromosomal Aberrations Detected by CGH

Aberration	Histological Subtype				HPV Type			
	SCC	Adeno	Adeno-SCC	Small Cell	16	18	Other	Negative
3q+	9	4	1	1	4	7	3	1
1q+	3	4	1		2	4	2	
Xq+	5	3			5	3		
20q+	1	3	2	1	1	5	1	
3p−	6				4	1	1	
20p+	1	3		1	1	4		
Xp+	2	2	1		2	3		
1p+	1	3			1	2		1
10q−	2	2			3		1	
11q−	3	1			2		2	
13q+	2	2			2	2		
5p+	3				1	2		
5q−	2			1	1	2		
6p−	2	1			2	1		
6q−	3				2	1		
8q+	3				3			
9p+	2		1		1	2		
15q+	1	2			2		1	
16q+	3				2			1
17p−	1	2			1		1	1
Xp−	1	1		1	1	2		
Xq−	1	1		1	1	2		

indicated no correlation of 5p gain with the level of telomerase activity or with telomere length measurement.

There have been several studies examining the chromosomal aberrations that occur in primary cervical tumors or cervical tumor cell lines. These studies have used a variety of techniques, including classical cytogenetics, LOH, and CGH. Aberrations of chromosomes 1, 3, and 5 were the most commonly reported changes. Chromosome 3p LOH has been reported in several studies of cervical carcinoma and 3q gain the most common aberration detectable by CGH. This aberration was suggested to define the transition from severe dysplasia to invasive carcinoma of the cervix (9). Isochromosome 5p and 5p gain have also been identified as aberrations characteristic of cervical carcinomas. Few of these studies have compared the histologic subtype of the tumors with the chromosomal aberrations detected in the tumors.

In this study we found chromosome 3q gain to be the most common chromosomal aberration detected by CGH of the primary cervical tumor samples. However, this aberration was not restricted to a specific histologic subtype. As stated above we also detected 3p loss in several tumors, and these aberrations, unlike those of 3q, were detected in only squamous cell carcinomas. At least four distinct regions of LOH have previously been mapped to chromosome 3p in solid tumors, including cervical carcinomas (25). These regions include 3p12, 3p14, 3p21, and 3p24–25, and are putative sites of tumor suppressor genes.

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