Advances in Brief

Genetic Alterations Accumulate during Cervical Tumorigenesis and Indicate a Common Origin for Multifocal Lesions

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Abstract

Carcinomas of the uterine cervix are thought to arise from preinvasive dysplastic lesions, termed cervical intraepithelial neoplasias (CIN), grades I–III. Patients may present clinically with two or more distinct lesions of differing histological severity; however, the genesis of these multifocal lesions is unknown. Despite infection with high-risk human papilloma virus subtypes, which is a major etiological factor in disease pathogenesis, only a small and unpredictable number of dysplastic lesions progress to invasive cancer. Several lines of evidence suggest that additional somatic events, such as tumor suppressor gene inactivation, are required for malignant transformation. In support of this, loss of heterozygosity (LOH) analyses of invasive cervical carcinomas have identified several chromosomal arms likely to harbor tumor suppressor genes, of which regions on chromosomes 3p, 4p, 4q, and 11q have been validated extensively. To evaluate the somatic arms likely to harbor tumor suppressor genes, of which regions on analyses of invasive cervical carcinomas have identified several chromosomal arms likely to harbor tumor suppressor genes, of which regions on chromosomes 3p, 4p, 4q, and 11q have been validated extensively. To evaluate the potential role of tumor suppressor gene inactivation in dysplastic progression, loci distributed on these four chromosomal regions were assessed for LOH in 42 CIN lesions of varying histological grade obtained from 17 patients. Analysis of at least 16 microsatellite loci in each lesion revealed allelic losses involving one or more of these chromosomal regions in 0% of CIN I lesions; 25% of CIN II lesions; and 88% of CIN III lesions, with 41% of CIN III lesions exhibiting LOH for three or more chromosomal regions. In addition, where LOH was scored for the same locus at a particular chromosomal region in all of the multiple lesions from a single patient, the same allele was lost at each locus, without exception. Statistical analysis of these allele-specific losses strongly suggests that topologically distinct lesions are related and likely arise from a common precursor cell.

Introduction

Premalignant lesions of the uterine cervix, termed CIN, grades I–III, represent a pathological continuum of mild to severe epithelial dysplasia (1, 2). Pathological and epidemiological examination of the natural history of CIN lesions shows that if left untreated, cervical dysplasia of all grades can spontaneously regress, persist, or, in a minority of cases, progress to invasive cancer (2). Currently, it is difficult to predict the clinical behavior of an individual precancerous lesion, although the presence of high-risk HPV sequences is indicative of a substantially increased relative risk for cervical dysplasia and invasive carcinoma (CC; Ref. 3). The oncogenic potential of HPV appears to be mediated by the E6 and E7 proteins of high-risk HPV subtypes, which are known to bind and inactivate p53 and pRB, respectively (4, 5). As a result, infected cells are likely predisposed both to the accumulation of DNA damage following carcinogenic insult (6) and increased rates of cellular proliferation. Because only a minority of HPV-positive lesions progress to invasive cancer and generally do so over a long period of time, additional somatic events, which may include the functional inactivation of one or more tumor suppressor genes, are almost certainly required for malignant transformation. Numerous studies based on somatic cell, cytogenetic, and LOH analyses strongly support this notion and have led to the localization of putative tumor suppressor genes on several chromosomes, such as 3p (7–10), 4p, 4q (11, 12), 5p (13), and 11q (14–17), among others (18–20). Two studies, in addition, have included a limited number of dysplastic samples and have reported allelic losses on chromosomes 3p (8) and 5p (13), implying that loss of function for tumor suppressor genes may occur early during cervical carcinogenesis. However, it is not clear if these losses are related to certain CIN phenotypes or how many such genes might be involved.

In a significant number of cases, patients diagnosed with cervical dysplasia present with multiple lesions of varying histological severity. Serial section analysis may reveal a single, deep lesion that has diffused laterally with focal extensions to the epithelial surface or, in many cases, may reveal multiple lesions that are distinctly separate entities. The genesis of these multifocal lesions is not understood but could conceivably arise as the result of a generalized "field effect," whereby continuous carcinogenic insults, such as HPV infection, may give rise to multiple, independently dysplastic cells. Alternatively, these lesions may be genetically related, having arisen from a single parental lesion and spread by cell migration to distant sites in the ectocervix.

To investigate the possible role of genetic alteration in the genesis and progression of cervical dysplasia and to understand the origin of multifocal cervical disease, 42 cervical lesions of varying histological grades derived from 17 patients were examined for allele-specific losses. Twenty-four microsatellite loci were chosen to target regions on chromosomes 3p, 4p, 4q, and 11q, which are known to undergo frequent alteration in invasive CC. In addition, the presence of HPV sequences and the specific subtypes involved were determined for each lesion and compared to patterns of observed LOH.

Materials and Methods

Patient Materials. Sixteen patients with two or more topologically distinct CINs and one patient with a single CIN II lesion were identified retrospectively from pathology reports filed between January 1994 and December 1995 at St. Joseph Hospital (Orange County, CA). The physical location of individual lesions within distinct quadrants of the cervix was confirmed by review of the colposcopy reports filed in each patient’s medical record. A total of 42 formalin-fixed and paraffin-embedded lesions classified as 13 CIN I, 12 CIN II and 17 CIN III were selected for analysis. The average number of lesions per patient was 2.5.

Enrichment of Dysplastic Lesions and DNA Preparation. Fifty to 100 consecutive sections (4 μm) of dysplastic tissue were cut from each block, and the first, middle, and final sections were stained with H&E and pathologically

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3 The abbreviations used are: CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; CC, cervical carcinoma; LOH, loss of heterozygosity; CIS, carcinoma in situ.

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examined. Disease margins were marked on H&E-stained sections and used as a guide for the removal of normal, contaminating tissue using sterile scalpels and a ×10 dissecting microscope. After enrichment, dysplastic cells were removed from the slides using another scalpel and pooled. Normal, control tissues were scraped from slides as described above. DNA was prepared according to Mashal et al. (21) with slight modifications. Specifically, all reagent volumes were decreased, proteinase K digestions were extended to 48–72 h, and DNA concentrations were determined by fluorimetry (Hoffman Scientific Instruments) prior to PCR.

PCR. Template DNAs (2–10 ng/reaction) were amplified for 35–40 cycles with AmpliTaq Gold (Applied Biosystems, Division of Perkin-Elmer, Foster City, CA) using the "AmpliTaq Gold linkage mapping set" conditions recommended by the manufacturer. PCR products were analyzed by electrophoresis on an ABI model 373 sequencer (Applied Biosystems).

LOH Analysis. DNAs from normal tissue of each of the 17 patients were first tested for heterozygosity at 24 microsatellite loci distributed on chromosomes 3p, 4p, 4q, and 11q (see Fig. 1) using six multiplex marker sets of four fluorescently labeled loci each. Homozygous, noninformative loci were replaced with other closely mapping loci to create four multiplex marker panels individualized for each set of multifocal dysplasias. Following amplification and electrophoresis, allelic products were assessed for peak height and peak area using GENESCAN and Genotyper (Applied Biosystems), and allelic ratios of heterozygous normal and lesion alleles were calculated as described previously (12, 22). LOH was assessed in all 42 CIN lesions at least two times independently; critical breakpoints were confirmed using coamplification of loci exhibiting LOH and nonsyntetic loci showing retention of heterozygosity.

Identification of HPV Subtypes. Sequences of the HPV L1 gene were detected using the MY09/MY11 primers (23). Ten to 20 μl of PCR product from positive lesions were digested with 10 units of Rsal (Stratagene, La Jolla, CA), and digestion products were visualized on 3% Metaphor agarose gels. Rsal digestion allowed unequivocal determination of HPV subtypes 6b, 11, 16, 18, and 33. For L1 products with unknown digestion patterns, full-length PCR products were isolated from the gel and directly sequenced using the MY11 primer on an ABI 373 or 377 automated DNA sequencer. Sequences were searched for database similarities using BLASTn. The HPV subtype was identified in each case with greater than 98% identity over a stretch of 300 to 350 nucleotides.

Results

Analysis of LOH in Cervical Dysplasia. Based on the observation of frequent LOH on chromosomes 3p, 4p, 4q, and 11q in primary CCs (7–10, 12, 17, 18), 24 markers mapping within and flanking the most commonly altered regions on these chromosomal arms were chosen to evaluate LOH in DNAs prepared from 42 preinvasive lesions representing the entire histological spectrum of cervical dysplasia. To maximize the informativeness of this analysis, normal control DNAs from the 17 patients with multifocal disease were evaluated for heterozygosity at all 24 loci. Noninformative loci were replaced with closely mapping informative loci. Overall, the 42 lesions were assessed with an average of 16 loci distributed on each of the three chromosomes (Fig. 1). No evidence of LOH was found in any of 13 CIN I lesions. However, LOH for loci on one or more of the chromosomal regions examined was detected in 25% of CIN II (3 of 12) and 88% of CIN III lesions (15 of 17). This accumulation of genetic alterations is exemplified in Fig. 1 (Case 11), which shows the comparison of a CIN II and CIN III from patient 11; LOH is seen only in the more severely dysplastic lesion. LOH was most frequently detected with loci on 3p; specifically, allelic loss was scored in 3 of 12 cases of CIN II (25%) and in 14 of 17 cases of CIN III (82%). The next most frequent alterations were observed on 11q, followed by 4q and 4p, respectively. A comparison of the cumulative LOH data in


Allele-specific Losses in Multifocal Cervical Lesions. A comparison of LOH patterns in multiple lesions from the same patient revealed several cases where the same locus or group of loci showed LOH (Fig. 1B). Without exception, LOH in each of the multiple lesions involved the same allele from each locus. This is exemplified in Fig. 3, which shows identical, allele-specific LOH for loci on chromosomes 3p, 4q, and 11q in three topologically distinct CIN III lesions (Fig. 3). Theoretically, for any locus that shows LOH, there is a 0.5:0.5 chance of observing loss from either the paternal or maternal allele in a heterozygous individual. The probability, P, of observing loss of the same allele in multiple, topologically distinct lesions is $0.5^{n-1}$, where n is the number of lesions. In case 13, for example (Fig. 1B), distinct regions of LOH occurred on 3p24.3, 3p14.1, 4q, and 11q. Each of these genetic events is probably independent, based on our previous observations in primary cancers that showed that losses on each of these three chromosomal arms can occur in any combination (0, 1, 2, or 3 losses), and in addition, LOH for multiple regions on the same chromosome can also occur in any combination (12, 17).

Therefore, in case 13, the probability of loss of the same allele for loci on all four chromosomal regions is $P = (0.25)^n$ or 0.004. Of 17 patients, multifocal lesions from four cases, 13, 14, 16, and 17, exhibited allele-specific losses with individual probabilities of 0.004, 0.125, 0.25, and 0.125, respectively. The overall likelihood of observing these allele-specific losses by chance alone is the product of the individual probabilities, $P = 1 \times 10^{-5}$. This strongly suggests that topologically distinct cervical lesions are genetically related and arise from at least a very few precursor cells.

Human Papilloma Viral Subtypes in Cervical Dysplasia. HPV subtypes were typically determined by PCR amplification of the L1 gene followed by restriction digestion with Rsal. In some cases, the L1 PCR product was directly sequenced, and in other cases, the subtype identity was confirmed using both methods. Of the 42 lesions tested, HPV sequences were found in 36 (84%). The specific HPV subtypes determined for each lesion are shown in Table 1. As expected, many lesions were infected with HPV subtypes that are commonly found in dysplasia and invasive CC (i.e., types 16, 31, and 33; Ref. 1). However, others, such as patients 2 and 11, were infected with HPV subtypes 54 and 56, respectively, which are found more rarely. With two exceptions (patients 7 and 14), multiple lesions from the same patient were infected with the same HPV subtype. Both aggressive and nonaggressive HPV types were observed in CIN I lesions, whereas in contrast, the majority of HPV positive CIN II lesions (11 of 12) and all HPV-positive CIN III lesions were found to be infected with aggressive subtypes, consistent with other reports (1, 24).

Discussion

Prospective clinical studies of patients with cervical dysplasia show that about 15% of intraepithelial neoplasias grades I–III are likely to progress to CIS, with progression from CIS to invasive cancer occurring in about 36% of cases. Overall, however, less than 2% of CIN lesions of all grades are likely to progress to invasive cancer, as evidenced by worldwide epidemiological studies (2). In comparing the biological characteristics of CIN phenotypes, several parameters are more commonly associated with severe dysplasia: (a) infection of the cervical epithelium with high-risk HPV subtypes (25); and (b) increased DNA index and the numbers of detectable aneuploid clones

5 A. A. Larson and G. M. Hampton, unpublished observations.
distinct entities: a group of polyclonal lesions generally associated with nonaggressive HPV subtypes (probably productive viral infections); and a group of monoclonal lesions, generally associated with aggressive HPV subtypes (24). Of the 9 CIN I lesions containing HPV sequences, 7 were infected with low-risk subtypes and, therefore, probably represent reactive, polyclonal proliferations. At least in this study, therefore, we cannot draw any conclusions with respect to LOH in mild dysplasia on chromosomes 3p, 4, and 11q.

Among the 13 CIN I lesions examined in this study, no evidence of LOH was found for any of the loci examined, which may, in part, be explained by the fact that CIN I lesions appear to be composed of two distinct entities: a group of polyclonal lesions generally associated with nonaggressive HPV subtypes (probably productive viral infections); and a group of monoclonal lesions, generally associated with aggressive HPV subtypes (24). Of the 9 CIN I lesions containing HPV sequences, 7 were infected with low-risk subtypes and, therefore, probably represent reactive, polyclonal proliferations. At least in this study, therefore, we cannot draw any conclusions with respect to LOH in mild dysplasia on chromosomes 3p, 4, and 11q. In contrast, LOH was observed in 3 of 12 CIN II lesions (25%). Eleven of the 12 CIN II lesions examined in this study were found to be infected with aggressive HPV subtypes and are likely to be monoclonal in origin and, therefore, evaluable for LOH. Thus, the numbers of LOH events reported here are likely to be an accurate reflection of the frequency of LOH on the chromosomal regions studied. A review of data reported here are likely to be an accurate reflection of the frequency of LOH in CIN II lesions (particularly for loci on chromosomes 3p, 4, and 11q) which are so similar. However, any potential correlation would need to be addressed with significantly larger numbers of samples and analysis of additional marker loci genome-wide. LOH was found to be very frequent in CIN III lesions, with LOH for one of 24 microsatellite loci used in this study. Colposcopy report diagrams with the four quadrant divisions of the cervix depicted for two patients (26, 27). These biological markers, alone or in combination, however, are not predictive of the behavior of an individual lesion. In an attempt to identify potentially early events that may correlate with dysplastic progression, we evaluated LOH at loci on chromosomes 3p, 4p, 4q, and 11q in 42 dysplastic lesions of all grades obtained from 17 patients.
or more loci occurring in 88% of cases, of which 41% showed LOH for three or more of the chromosomal arms examined. The incidence of LOH in CIN III lesions, in contrast to that seen in CIN II lesions, is higher than the estimated rates of progression of severe dysplasia and CIS (36%; Ref. 2). Thus, LOH, per se, does not appear to be a marker of progression to CIS and/or invasive CC but rather a marker of more severely dysplastic lesions.

The highest frequency of LOH was observed for loci on 3p, increasing in frequency from 25% in CIN II to 82% in CIN III. Alterations of 3p were also observed as the sole event in two of the three CIN III lesions with LOH for any of the loci examined. Thus, it appears that alterations on this chromosomal arm may precede alterations on chromosomes 4 and 11q. Three regions were commonly affected; 3pter–3p24; 3p14.2, and 3p14.1–12, which essentially agrees with our analyses of 3p losses in primary CC and CC-derived cell lines.4 However, our analyses of primary CC has shown that over

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**Fig. 2.** Accumulation of genetic alterations during cervical carcinogenesis. Graphical representation of LOH for loci on chromosomes 3p, 4p, 4q, and 11q in varying grades of dysplasia and selected stage IB invasive squamous cell carcinomas of the uterine cervix. Numbers in parentheses, total numbers of lesions/carcinomas examined. NCE, normal cervical epithelium; CxCa, invasive cervical carcinoma.

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**Fig. 3.** Identical patterns of allele-specific LOH in three CIN III lesions from a single patient. Shown in this figure are representative electropherogram traces from one locus on each of the three chromosomes examined, D3S1597, D4S1565, and D11S1391, in three cervical lesions from patient 13 (96496). The top three panels depict the heterozygous peaks for each of the three loci in the normal tissue from this patient. The next three sets of panels show the dysplasia-specific reductions of the second allele in the first two loci and the first allele in the third locus (arrows). The relative peak height for each allele is measured in fluorescent intensity units (fu); fu values are shown in the top left corner of each. The allelic ratios for each of the LOH events are shown in the top right corner of each panel. Note the identical loss of the same allele for each locus in each of the topologically distinct lesions.

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4174
GENETIC ALTERATIONS IN CERVICAL DYSPLASIA

Table 1  Clinical characteristics and HPV status of cases with multiple dysplastic lesions

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<th>Site</th>
<th>Histology</th>
<th>HPV type</th>
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<td>31</td>
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<td>D2</td>
<td>3 oc</td>
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<td>12 oc</td>
<td>CIN II</td>
<td>16</td>
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<td>2 oc</td>
<td>CIN II</td>
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<td>CIN III</td>
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* D: dysplasia.
* oc: o'clock position of colposcopic biopsy.
* Histology.
* HPV negative by L1 PCR assay.

Severe dysplasia is generally associated with an increase in DNA index and aneuploidy as compared to CIN II and CIN 1 lesions, respectively (26, 27). This increase in genomic instability is both consistent with the fact that the E6 protein of aggressive subtypes is known to bind p53 and mediate its degradation through a ubiquitin-dependent pathway (5) and with the observation that transfection of some 11 via microcell mediated chromosome transfer controls tumorigenic expression of E7 oncoprotein is able to bind the retinoblastoma gene product. Science (Washington DC), 243: 934—937, 1989.

70% of individual tumors exhibit loss of multiple, distinct regions of this chromosome, making the identification of tumor suppressor genes relevant to CC difficult.4 In contrast, the patterns of allelic loss in dysplasia were relatively simple. Thus, analysis of 3p losses in a larger number of CIN II/CIN III lesions with a larger number of loci may allow a clearer interpretation of the role of relevant tumor suppressor loci and lead to further genomic sublocalization. LOH on chromosome 4 in dysplasia supported our previous localization of putative tumor suppressor genes to 4p and 4q in primary cancers (12) and also allowed us to refine the position of these two genes to 4pter—D4S403 and D4S426—4qter.

Acknowledgments

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