Deletions of Chromosome 3p Are Frequent and Early Events in the Pathogenesis of Uterine Cervical Carcinoma

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Abstract

To study the molecular abnormalities involved in the multistage development of cervical carcinoma (CC), we investigated the presence of oncogenic human papillomavirus (HPV) sequences, loss of heterozygosity (LOH), and microsatellite alterations at several genes/loci at 3p (3p14.2 at the FHIT gene, 3p14.3–21.1, 3p21, and 3p22–24.2), 9p21, RB and P53, and P53 gene point mutations in precisely microdissected archival tissues from 20 CCs and their accompanying precursor lesions (cervical intraepithelial neoplasia, CIN; n = 40) and normal epithelia (n = 20). In all HPV-positive cases (90% of CCs), HPV sequences were detected as the earliest appearing molecular change or simultaneously with other changes. LOH at any 3p region was found in 70% of CCs, and 3p14.2 (FHIT gene/FRA3B fragile site) (56%) and 3p21 (57%) were the most frequent 3p sites of loss. LOH at some 3p region was in the CIN I stage, and the 3p deletions in the associated invasive CC. LOH at the other regions studied and P53 gene mutations were less frequent and later events. Microsatellite alterations were detected in 35% of CCs, and identical abnormalities were detected in the associated precursor lesions. Although infection with oncogenic HPV strains is the earliest and most frequent molecular event, progressive deletions at one or more 3p regions (particularly at 3p14.2, and 3p21) are also frequent events occurring early in the pathogenesis of CC.

Introduction

Although the death rate from CC is relatively low in the United States, CC is one of the most common malignancies in women in developing countries (1). Most CCs are squamous cell carcinomas (90%) and are preceded by precursor intraepithelial lesions (CIN; Ref. 1). Whereas the low-grade CINs (CIN I) have a low rate of progression to invasive cancer, the high-grade CINs (CIN II and III) have a much greater potential of progressing, and these lesions are considered high-risk precursors for development into CC (1). Many studies have linked the presence of specific types of human papilloma virus (HPV) with the development of CC (1, 2). However, HPV infection alone is probably insufficient for complete neoplastic transformation of the cervical epithelium (2). Multiple genetic changes, especially involving TSGs, are associated with the development of many human cancers. Inactivation of p53 and Rb proteins by E6 and E7 proteins from integrated oncogenic HPV strains is an important component of TSG inactivation in the vast majority of HPV-positive CCs (1). However, other genetic changes appear also to be required for malignant transformation in the pathogenesis of this neoplasm. Allelotyping studies in CC have revealed frequent LOH affecting many chromosomal loci, including several regions located on the short arm of chromosome 3 (3p) (3–7). Deletions at chromosome 3p are common in many other human cancers, and at least four distinct 3p regions, which include 3p12, 3p14, 3p21, and 3p24–25, are believed to harbor TSGs (8). Recent attention has focused on FHIT, a candidate TSG at 3p14.2 that spans FRA3B, the most common of the aphidicolin-inducible fragile sites (9). FRA3B is also a candidate region for HPV-16 integration (10). In addition to LOH, alterations in microsatellite size (MAs) are other genetic changes associated with several human cancers (11). The mechanisms underlying MAs are currently unknown, but they probably represent a form of genomic instability (11). With developments in microdissection of archival material, PCR techniques, and new polymorphic DNA markers, it is now possible to study the sequence of molecular changes in preneoplastic lesions spanning the multistage development of neoplasms. However, data about the role of other genetic changes besides HPV infection in the progression of CC are meager. Thus, using a precise microdissection methodology and archival paraffin-embedded tissue sections, we studied the presence of oncogenic HPV sequences, the incidence of LOH and MAs at several chromosomal regions at 3p (3p14.2 at FHIT gene, 3p14.3–21.1, 3p21, and 3p22–24.2), 9p21 (p16), 13q14 (RB), and 17p (P53), as well as P53 gene point mutations, in invasive CCs and their accompanying precursor lesions and normal epithelia.

Materials and Methods

Tissue Samples and Histological Diagnosis. Paraffin-embedded archival material from 20 early stage squamous cell CCs obtained by hysterectomy containing areas of precursor lesions (CINs, n = 40) and normal squamous epithelium (n = 20) were selected for study. The precursor lesions were classified into low grade (CIN I; n = 11) and high grade (CINs II and III; n = 29). We also identified areas of MICA in seven cases. MICA is considered a histopathological stage in the progressive spectrum, between high grade CIN and frank clinically invasive CC (1). We used the criteria of the WHO and International Society of Gynecological Pathologists for histological identification of pathological lesions (1).

Microdissection and DNA Extraction. Areas of normal and abnormal epithelia (CIN), as well as invasive carcinoma, were identified and precisely dissected under microscopic visualization (Fig. 1), and DNA extractions were performed as described previously from non-cover-slipped, H&E-stained slides (Fig. 1; Ref. 12). Microdissected stromal cells from the same cases provided a source for constitutional DNA. From multiple sections of each tissue sample, 300–1000 sectioned cells were microdissected. Five μl of the proteinase K-digested samples, containing DNA from at least 50 sectioned cells, were used for each PCR reaction.

Polymorphic DNA Markers and PCR Analyses. To evaluate LOH and MAs, we used primers flanking dinucleotide and multinucleotide microsatellite repeat polymorphisms located at the following genes or chromosomal loca-
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Fig. 1. Representative examples of microdissection of invasive squamous cell cervical carcinoma and high-grade precursor lesion. Note in invasive carcinoma (a, before and b, after microdissection) that only malignant cells were microdissected, and in precursor lesion CIN II (c, before and d, after microdissection), the basal membrane is intact. a and b, H&E X75; c and d, X50.

Fig. 2. Representative gel and autoradiographs of HPV and microsatellite analyses of six cases of invasive cervical carcinomas and accompanying normal epithelium and precursor lesions (CIN) showing LOH at several chromosomal regions at 3p. N, normal squamous epithelium; CIN I, low-grade cervical intraepithelial neoplasia; CIN II and III, high-grade intraepithelial neoplasias. Bars, the position of the major allelic bands for LOH studies.

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MUTATIONS: 3p14.2 (FHIT gene locus), 3p14.3–21.1, 3p21, 3p22–24.2, 9p21, 13q14 (RB gene), and 17p13.1 (P53 gene). The markers used for chromosome 3p regions analyses are listed in Fig. 3, and the probes used to test the other loci are the following: IFNA and D9S171 at 9p21; dinucleotide and tetrancleotide repeats in introns 2 and 20 of the RB gene; and dinucleotide (TP53) and pentancleotide repeats in the P53 gene. The primer sequences used for LOH studies were obtained from the Genome Database with the following five exceptions: (a) RB-tetrancleotide (13); (b) RB-dinucleotide (14); (c) P53-pentancleotide (13); (d) P53-dinucleotide (15); and (e) ITIH-1 (16). Nested PCR methods were used to amplify dinucleotide and multinucleotide repeats from microdissected cells as described previously (17). LOH was scored by visual detection of complete absence of the upper or lower allele (Fig. 2). MAs were detected by a shift in the mobility of one or both alleles compared to constitutional DNA (from normal stromal cells) from the same individual.

Detection of HPV Sequences. The presence of HPV sequences was tested by PCR using general and type-specific primers designed for paraffin-extracted DNA (17, 18). Specific primers were used to identify high (HPV 16 and 18) and intermediate (HPV 31 and 33) oncogenic risk HPV strains (18). The HPV analysis was done using one-round PCR. DNA extracted from human cell lines CaSki (HPV 16) and HeLa (HPV 18; obtained from the American Type Culture Collection, Rockville, MD) were used as positive controls for HPV analyses.

P53 Gene Mutation Study. We modified a method published previously for application to archival tissues (19). We examined for mutations in exons 5–8 of the P53 gene using a nested PCR methodology, followed by SSCP analysis. As degradation of DNA by formalin fixation limits reproducible amplification of DNA to fragments smaller than 250 bp, the primers were designed to bracket relatively short DNA fragments between 100 and 200 bp. Because exon 5 is relatively large, two sets of primers were used to amplify it.
Abnormal SSCP bands were isolated and sequenced. The PCR conditions for SSCP analyses were the same as that for LOH. Abnormal SSCP bands were isolated and sequenced. One of the oligonucleotide primers used in generating PCR products for sequencing from cut bands was biotinylated, enabling the isolation of single-stranded DNA by streptavidin-coated Dynabeads. The single-stranded DNA was then sequenced using a sequencing kit (T7 Sequenase, version 2.0; Amersham Life Science, Cleveland, OH). Both the sense and antisense strands were sequenced to confirm the presence of a mutation.

Statistical Analysis. The cumulative binomial test was used to examine the likelihood that the occurrence of a particular event (loss of the same allele in the invasive carcinoma and its corresponding preneoplastic lesions) occurs at a particular probability when observed in repeated trials. When the results are compared with a chance occurrence or non-occurrence, the particular probability of comparison is 0.5.

Results

Detection of HPV Sequences. Eighteen of 20 (90%) CCs were positive for oncogenic HPV sequences using general and type-specific HPV primers (Table 1 and Fig. 3). The HPV type distribution was as follows: 12 cases (60%) of HPV-16, 4 cases (20%) of HPV-18, 1 case (5%) of HPV-31, and 1 case (5%) of HPV-33. Identical type-specific sequences were detected in corresponding precursor lesions: in 29 of 40 (73%) CINs and in 1 of 20 (5%) normal squamous epithelium (Table 1 and Fig. 2). HPV sequences were not detected in normal or abnormal epithelia associated with HPV-negative CCs.

LOH of Chromosome 3p Arm Regions in Cancer and Precursor Lesions. Fourteen of 20 (70%) invasive cervical carcinomas showed LOH at one or more of the chromosome 3p regions analyzed (Table 1; Figs. 2 and 3). The 3p deletions detected in invasive tumors were in some cases extensive (3 cases, 15%), but in the majority of the cases, the deletions were localized (Fig. 3). Because most of the CCs...
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demonstrated HPV sequences, no correlation between 3p LOH and HPV status could be established. However, the two HPV-negative CCs also showed LOH at 3p loci (Fig. 3), and the 3p allelic loss in these cases was also detected in accompanying early stage (CIN I) lesions. A high incidence of LOH was detected at 3p14.2 (FHIT gene; 9 of 16, 56%) and 3p21 (8 of 14 cases, 57%) regions in CCs (Table 1 and Fig. 3). Allelic losses at 3p14.3–21.1 (5 of 13 cases, 39%) and 3p22–24.2 (6 of 20 cases, 30%) regions were less frequent. There was no consistent shortest region of overlap found for LOH. Instead, it appeared that several 3p regions (3p14.2, 3p14.3–21, 3p21, and 3p22–24) could undergo localized LOH.

LOH at one or more 3p regions was a frequent event (14 of 25 lesions, 56%) in precursor CINs lesions accompanying the invasive CCs (Fig. 2). There was a progressive increase of the LOH incidence at one or more 3p regions, in precursor lesions with increasing severity of histopathological changes (Table 1), although the number of specimens were too small for statistical analysis. Although LOH at 3p14.3–21.1, 3p21, and 3p22–24 regions was detected at the earliest precursor stage of low-grade lesion (CIN I), allelic loss at 3p14.2 was found only in high-grade precursor lesions (CIN II and III). Allelic loss (at any 3p region) was not detected in any normal squamous epithelia.

We compared the size of the chromosome 3p deletions between invasive carcinomas and their accompanying microinvasive and precursor CIN lesions in cases in which one or more 3p regions analyzed demonstrated LOH in tumors. In all three microinvasive cancers, identical deletions were present as in the corresponding invasive tumors. However, the deletions in CIN lesions were usually smaller in extent that in the corresponding invasive cancers (9 of 16 comparisons, 56%).

Frequency of Other Molecular Changes. P53 gene abnormalities (either point mutations or LOH) were less frequent events in CC (6 of 20, 30% of the cases). Although P53 point mutations were detected in 3 of 20 (15%) carcinomas (data not shown), allelic loss at the P53 gene was demonstrated in 4 of 15 (27%) of the tumors (Table 1). Only a single case demonstrated both allelic loss and a point mutation. Although P53 point mutations were a late event (invasive carcinoma), allelic loss was an early change in a subset of cases (CIN I). In the invasive carcinoma in which both P53 gene LOH and point mutation were detected, allelic loss without mutations was found in accompanying precursor CIN I lesions. In contrast to expectations, the two HPV-negative CCs lacked p53 gene abnormalities.

Although LOH at chromosome 13q (RB gene; 25%, 5 of 20 informative cases) and 9p21 (11%, 2 of 19 informative cases) regions were infrequent abnormalities in CCs, allelic loss was detected at both loci at the stage of high-grade precursor lesions (CIN II and III; in a subset of cases).

Frequency of MAs. We found MAs in 7 of 20 (35%) CCs. Six of these alterations were seen in chromosome 3p loci and one in RB gene. In the cases with MAs in the invasive tumor, the same abnormality was detected in MICA (two of three cases) and in both grades of precursor lesions (CIN I, two of four cases; CIN II and III, three of eight). MAs were not detected in normal epithelium (n = 9) accompanying invasive tumors.

Patterns of Genotypic Changes. From the above-mentioned results, three patterns of genotypic changes in invasive CCs could be discerned: (a) cases with HPV sequences and 3p deletions (12 cases, 60%); (b) cases with HPV sequences and without other detected molecular change (6 cases, 30%); and (c) cases with 3p deletions but without HPV sequences or other molecular changes (2 cases, 10%). MAs were present in seven cases, all of which had 3p deletions, including the two cases without HPV sequences.

Sequential Changes during Multistage Carcinogenesis. The presence of HPV sequences of high- and intermediate-risk strains was the earliest molecular change detected during the multistage development of CC (Table 1 and Fig. 2). HPV sequences were detected in one example of normal epithelium and increased in frequency with progressive histological change. In all 18 HPV-positive cases, HPV sequences were detected as the earliest appearing molecular change (9 cases, 50%) or simultaneously with other changes (9 cases, 50%). In addition, HPV sequences were demonstrated in precursor lesions without 3p LOH at an earlier stage of CC pathogenesis (6 of 10 cases) or appeared simultaneously with 3p LOH (4 of 10 cases).

The next most frequent and earliest change (commencing at CIN I) was LOH at one or more 3p regions. LOH at chromosome 3p regions was detected at earlier stages of the development of CC (7 of 10 cases, 70%) than P53 gene abnormalities (either point mutation and LOH) and LOH at the other chromosomal regions analyzed (9p21 and RB gene).

Molecular Changes in Precursor Lesions Are Identical to Those in Corresponding Invasive Cancers. The same HPV strain was present in normal epithelium and precursor lesions as in the corresponding invasive cancers (27 of 27 comparisons). We compared the specific parental allele lost in the invasive carcinoma with the parental allele lost in the precursor lesions. For all comparisons, the same parental allele was lost in 40 of 43 comparisons (93%). The possibility of this happening by chance is remote as tested by the cumulative binomial test (P = 1.5 x 10^{-6}). Similarly, an identical MA pattern was found in all comparisons (7 of 7) between invasive carcinoma and precursor lesions.

Discussion

We studied molecular abnormalities (oncogenic HPV sequences, LOH, and MAs at seven lociigenes, and P53 gene mutations) in precisely microdissected tissue from archival paraffin-embedded material from 20 cases of CC and their accompanying precursor lesions (CIN) and normal squamous epithelia. We detected HPV sequences in 90% (18 of 20) of the CCs, 73% of CINs, and 5% of normal epithelia. High risk HPV strains 16 and 18 were the most frequent (16 of 18, 89%) present. In all HPV-positive cases, HPV sequences were detected as the earliest appearing molecular change occurring alone or simultaneously with the LOH changes.

Our results indicate that LOH occurs frequently on the short arm of chromosome 3 (3p) in CC (70%). Other studies have found 3p LOH frequencies of 35–70% (3–7). Most of our CC cases (85%) demonstrated restricted regions of 3p LOH in contrast to the more extensive losses present in lung cancers (8). In addition, the 3p deletions in precursor CIN lesions were less extensive than the losses in corresponding microinvasive and invasive carcinoma, indicating that a progressive loss of putative 3p recessive oncogenes accompanies progressive histological changes. Of the four regions tested on 3p, those showing the highest frequency of LOH were 3p21 (57%) and 3p14.2 at the FHIT gene locus (56%). Abnormalities of the FHIT gene have been described in many tumors, although to date no data exist for cervical cancers.

Of interest, our results indicate that 3p LOH also occurs as an early event in the multistage development of this neoplasm, although it follows the presence of HPV sequences. Although LOH at 3p14.3–21.1, 3p21, and 3p22–24 regions was detected at the earliest precursor stage of low grade lesion (CIN I), allelic loss at 3p14.2 was found only in high-grade precursor lesions (CIN II and III). In CC, a small percentage of precursor lesions develop into invasive carcinoma after a long latency period (1). Most of the low-grade precursor lesions (CIN I) regress or persist for years, whereas the high-grade lesions (CIN II and III) have a higher rate of progression to invasive CC. It is possible that cervical precursor lesions having multiple molecular changes are at increased risk of progressing to invasive CC. Of interest, MICA, which may be regarded as the earliest invasive lesion, contained similar molecular changes as the more deeply invasive areas.
In all grades of cervical precursor lesions (CINs), the HPV genome usually is maintained as an episome, whereas in most of the invasive tumors the viral genome is stably integrated into the host cell DNA (20). Integration sites for HPV have been reported to be near fragile sites, oncogenes, and/or genes for transcription factors (10, 21, 22). With in situ hybridization, it has been shown in a primary cervical carcinoma that 3p21 is a target site for HPV-16 integration (21). More recently, an insertion site for the HPV-16 was found in close proximity to the FRA3B fragile site, which lies within the FHT7 gene (10). Because oncogenic HPV sequences were detected in the majority (90%) of our CC cases, no correlation with 3p LOH could be established. However, most of the CCs with 3p LOH demonstrated oncogenic HPV sequences (86%). In addition, HPV infection occurred either earlier (60%) or simultaneously (40%) with 3p LOH. The important role of 3p genes was emphasized by the finding that two tumors lacking HPV infection and the other molecular changes that we tested for contained allelic loss at one or more 3p regions.

Inactivation of p53 and Rb proteins by formation of complexes with E6 and E7 proteins, respectively, of high-risk oncogenic HPV strains (16 and 18) may be the major mechanism of inactivation of these genes during CC pathogenesis (1). Thus, we found a modest incidence of p53 and Rb gene nuclear abnormalities, similar to previous reports (23,24). Of interest, of the six CCs with any p53 gene abnormality, only one case had both LOH and a point mutation. These findings suggest that inactivation of even one p53 allele may reduce the amount of wild-type p53 protein required to be inactivated by E6. An alternative hypothesis is that there is another TSG located in the 17p13 region.

Allelic losses in the short arm of human chromosome 9 have been identified in several cancer types, and a putative oncogene has been identified (25). The gene, known by several names, including CDKN2, encodes the p16 protein. The low incidence of LOH at the 9p21 locus (11%) detected in our cases indicates that this abnormality is not an important event in the pathogenesis of CC.

Alterations in MAs are another genetic change associated with many cancers. The relationship of MAs to the DNA repair mechanism has not been established, but they probably represent evidence of some form of genomic instability (11). Nevertheless, MAs are attractive candidates for the early molecular detection of cancer (26). We found MAs in 35% of the CCs. Of interest, in the tumors with MAs, the same pattern of alteration was detected in their precursor lesions at early stages (CIN I) of the pathogenesis of CC.

Our findings suggested three distinct patterns of genotypic changes in invasive CCs. The most frequent (60%) was the presence of HPV sequences and 3p deletions, with or without varying numbers of the other changes. The next most frequent pattern was HPV sequences without other changes (30%). The least frequent pattern was 3p deletions without other changes, including lack of HPV sequences. These latter subgroups may contain changes in genes or chromosomal regions that we did not study. MAs were detected in 35% of cases, all of which had 3p deletion, suggesting that 3p deletions may be associated with genomic instability. These findings suggest a pivotal role for 3p deletions in the pathogenesis of most cases of cervical cancer.

The finding of identical patterns of molecular changes (HPV strain, allele specific loss, and MAs) in the invasive carcinoma and accompanying preeplastic lesions strongly suggests that the precursor lesions that we studied were clonally related to the corresponding invasive cancers. In other organs, such as the respiratory epithelium, other explanations for allele-specific loss may exist (12). However, in the cervix, the precursor lesions are in close anatomical proximity to the tumor and are more likely to be clonally related.

In summary, although HPV infection with high-risk strains of HPV is probably the earliest and most important event, progressive deletions at one of more regions at 3p (particularly 3p14.2 at the FHTI gene and 3p21) are also frequent and early events in the pathogenesis of CC.

References

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