DNA Aneuploidy and Integration of Human Papillomavirus Type 16 E6/E7 Oncogenes in Intraepithelial Neoplasia and Invasive Squamous Cell Carcinoma of the Cervix Uteri

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ABSTRACT

Purpose: Increasingly deregulated expression of the E6-E7 oncogenes of high-risk human papillomaviruses (HR-HPVs) has been identified as the major transforming factor in the pathogenesis of cervical dysplasia and derived cancers. The expression of these genes in epithelial stem cells first results in chromosomal instability and induces chromosomal aneuploidy. It is speculated that this subsequently favors integration of HR-HPV genomes into cellular chromosomes. This in turn leads to expression of viral cellular fusion transcripts and further enhanced expression of the E6-E7 oncoproteins. Chromosomal instability and aneuploidization thus seems to precede and favor integration of HR-HPV genomes.

Experimental Design: To prove this sequential concept, we analyzed here the sequence of events of DNA aneuploidization and integration in a series of HPV-16-positive cervical dysplastic lesions and carcinomas. Eighty-five punch biopsies of HPV-16-positive cervical lesions (20 CIN1/2, 50 CIN3, and 15 CxCa) were analyzed for DNA ploidy by DNA flow cytometry and for integration of HPV E6/E7 oncogenes using the amplification of papillomavirus oncogene transcripts assay, a reverse transcription-PCR method to detect integrate-derived human papillomavirus oncogene transcripts.

Results: DNA aneuploidy and viral genome integration were both associated with increasing dysplasia (P < 0.001, χ2 test for trend). In addition, DNA aneuploidy was associated with increased viral integration (P < 0.01, Fisher’s exact test). Nineteen of 20 (95%) lesions with integrated viral genomes had aneuploid cell lines; however, only 19 of 32 (59%) lesions with aneuploid cell lines had integrated viral genomes.

Conclusions: These data support the hypothesis that aneuploidization precedes integration of HR-HPV genomes in the progression of cervical dysplasia. Accordingly, deregulated viral oncogene expression appears to result first in chromosomal instability and aneuploidization and is subsequently followed by integration of HR-HPV genomes in the affected cell clones.

INTRODUCTION

Genetic instability is a hallmark of most malignant tumors. It occurs early in the development of precancers and enables them to develop a growing reservoir of proliferating cells that stepwise acquire mutations that permit unrestricted growth of cancer cells (1). In most cancers, genetic instability is achieved by alterations of the mitotic spindle apparatus that usually are caused by multipolar mitoses and an increasing disequilibrium in distribution of the chromosomes. Because of the severe numerical and structural changes of the chromosomes, this type of genetic instability is referred to as chromosomal instability (2). Because of the acquired increasing disturbances of cell cycle regulation, resistance to apoptosis, and vascularization, these cells might expand locally and create an increasing reservoir of cells with an abnormal structure and number of their chromosomes. The structural and numerical chromosomal changes result in an increasing shift of the overall DNA content of the cells, a phenomenon commonly referred to as aneuploidy. In cervical intraepithelial neoplasia (CIN) and invasive cervical cancer (CxCa), DNA ploidy estimation has been established as a prognostic factor that allows to estimate the relative progression risk into more advanced lesions (3–5).

Persistent infection with high-risk types of the human papillomavirus (HPVs) are the major risk factor for the development of cervical cancer (6). Two viral genes, E6 and E7, are continuously expressed in high-risk types of the HPV-transformed cells, and their expression is required to induce and maintain the neoplastic phenotype of cervical cancer cells (7). The E6-E7 gene products cooperate in disturbing cell cycle regulation, preventing apoptosis (8, 9), and inducing multiple mitotic aberrations, chromosomal instability, and nuclear aneuploidy (10, 11). Thus, cell clones that express the viral E6 and E7 oncogenes undergo chromosomal instability and rapidly develop aneuploidy.

Cervical cancer emerges from a series of histologically well-defined precursor lesions, referred to as CIN grades 1–3. The majority of low-grade dysplastic lesions regress spontaneously, and only a small percentage of lesions progress to severe dysplasia and finally cervical cancer (12, 13). The current view of cervical carcinogenesis suggests that in the initial events, high-risk types of the HPV-infected epithelial stem cells un-
Under specific changes that overcome the tight transcriptional control of viral gene expression in normal nontransformed epithelial stem cells (14). Inactivation of these cellular control functions permits deregulated transcription of the viral oncoproteins E6 and E7 from episomal HPV genomes that in turn confers chromosomal instability and provokes an increasing aneuploidization of the respective cells (11, 15). The increasing structural changes support the integration of foreign extra-chromosomal HPV genomes into chromosomes of the host cell. Some of the integrated genome fragments are transcribed, resulting in hybrid mRNA molecules that encompass viral sequences encoding the E6 and E7 gene products at the 5'-end and heterogeneous cellular sequences at the 3'-end (16). HPV DNA integration seems to be randomly distributed over the human genome with a preference for unstable chromosomal areas (17). Upon integration, intrinsic viral repressors of gene expression are lost (18), and cellular regulatory factors can additionally enhance HPV oncogene expression (19). Cotranscription of viral genes with cellular sequences contributes to the stability and thereby also transforming activity of the respective transcripts (20, 21). Thus, cells with integrated HPV genomes that express integrate-derived papillomavirus oncogene transcripts (iPOs) seem to gain strong growth advantages and are preferentially selected for clonal neoplastic outgrowth (22). This concept implies that aneuploidization occurs before integration of the viral genome in a sequential pathogenetic model. In recent in vitro studies, this sequential series of events has been demonstrated for high-risk types of the HPV-transformed keratinocytes (23, 24). To test whether this model is also valid for naturally occurring lesions, we have investigated the time course of aneuploidization and viral genome integration during the progression of cervical precancerous lesions.

MATERIALS AND METHODS

Tissue Sampling and Preparation. Eighty-five tissues samples, which were obtained from patients with HPV-16-positive CIN1/2 (n = 20), CIN3 (n = 50), and CxCa (n = 15) at the Colposcopy Clinic of the Women’s Hospital of the University of Heidelberg, were included in the study. They were part of colposcopically guided punch biopsies, which were taken for diagnostic purpose after written consent from suspicious lesions of the cervix uteri. One part was used for preparation of H&E-stained slides. The remaining part was shock frozen in liquid nitrogen and stored at −70°C. Fresh frozen sections were prepared before cytometric DNA analysis, HPV analysis and amplification of papillomavirus oncogene transcripts-PCR to confirm that tissues indeed contained the expected dysplastic lesions.

Flow Cytometric DNA Analysis. To determine the cellular DNA content, a standard protocol was applied (25). The tissues were minced and incubated in a detergent solution (0.1 M citric acid and 0.5% Tween 20; Serva, Heidelberg, Germany) with gentle shaking for 20 min at room temperature. After adding six volumes of a staining solution (0.4 M disodium hydrogen phosphate and 5 μM 4',6-diamidino-2-phenylindole; Partec, Münster, Germany), this mixture was stored for 24 h at room temperature before additional analysis. A PAS II flow cytometer (Partec) was used to analyze the samples. Lymphocytes were used to calibrate the system. The maximum permitted coefficient of variation for calibration was 2%. At least 10,000 cells were counted in each sample. DNA histogram cell cycle analysis was performed as described before (26), using Multicycle software (Phoenix Flow System, San Diego, CA). The G0-G1, S, and G2-M phases of the cell cycle were calculated. Cases were regarded as acceptable for analysis if the coefficient of variation of the G0-G1 peak was <7.0. Lesions were classified as diploid, tetraploid, or aneuploid (Fig. 1). If two distinct G0-G1 peaks were present with a DNA index of >1.15 (each containing >10% of total cell population), the histogram was considered aneuploid. By convention, the first G0-G1 peak (on the far left) represented the diploid peak. Diploid and tetraploid lesions were classified as nonaneuploid. Histogram patterns were termed diploid if there was a single G0-G1 peak. A histogram was classified as tetraploid only if a large peak in G2-M (DNA index of 1.80–2.2) made up >20% of the total curve and was also associated with a corresponding 8 N peak.

HPV Analysis. RNA from frozen tissues was isolated using a RNA isolation kit (ToTALLY RNA kit; Ambion, Inc., Austin, TX) as recommended by the supplier. DNA was extracted using an additional protocol for simultaneous extraction of RNA and DNA (Technical Bulletin 161; Ambion, Inc.).

Typing of HPV DNA was performed as previously described, using a standardized general primer mediated PCR system (GP5+/GP6+) with the colorimetric enzyme immunoassay read out system (27).

For amplification of papillomavirus oncogene transcripts, total RNA (1 μg) from HPV-16-positive probes was reverse transcribed and amplified using a protocol, as reported in detail previously (16). The final PCR products were electrophoresed in 1.2% agarose gels, blotted onto nylon membranes (Hybond N+, Amersham Life Science, Buckinghamshire, United Kingdom), and hybridized with a HPV-16 E7-specific probe. Labeling and detection of the probes was performed with the enhanced chemi-
The degree of neoplastic progression (DNA aneuploidy) was thus significantly linked to CIN2/3 and to 80% in the invasive cervical carcinomas. Prevalence of DNA aneuploidy ranged from 20% in the low-grade lesions (CIN 1) to 32% in the high-grade lesions (CIN 2/3). The percentage of aneuploid cell clones was determined in 32 of 85 cervical biopsies (Table 1).

Fig. 2 Amplification of papillomavirus oncogene transcripts (APOT) in three human papillomavirus type 16-positive CIN3 samples. The PCR products of ~1050 bp represent the abundant episomal transcripts (ePOTs). Amplimers with a different size represent integrate-type transcripts (iPOTs). Lane 1: CIN3 with positive detection of both ePOTs and iPOTs. Lane 2: CIN3 with positive detection of iPOTs. Lane 3: CIN3 with positive detection of ePOTs.

Association between DNA Aneuploidy and Integration of HPV-16 E6/E7 Oncogenes. Because both parameters, aneuploidy, and prevalence of iPOTs were associated with increasing grades of dysplasia of the HPV-16-positive lesions analyzed here, the question arose whether the iPOTs occur preferentially in aneuploid lesions, suggesting that aneuploidy favors integration of HPV genomes, or whether expression of iPOTs might be found preferentially in high-grade lesions that might not be in all cases aneuploidy, suggesting that in this scenario integration of papillomavirus genomes might precede and subsequently favor aneuploidy. To clarify this question, we compared the prevalence of DNA aneuploidy and iPOTs in the series of HPV-16-positive lesions. Nineteen of 20 (95%) of lesions that expressed iPOTs were aneuploid, whereas, however, only 19 of 32 (59%) of the aneuploid lesions expressed iPOTs (P < 0.001, Fisher’s exact test; Table 2). These data clearly demonstrate that almost all lesions with integrated papillomavirus genomes were aneuploid, whereas only a fraction of the aneuploid lesions expressed iPOTs.

RESULTS
Prevalence of DNA Aneuploidy and Transcription of Integrated HPV-16 E6/E7 Oncogenes. Eighty-five HPV-16 positive lesions, including 20 CIN1/2, 50 CIN3, and 15 invasive squamous cell carcinomas, were analyzed in this study. The age of patients from whom the samples were obtained was 33.1 years (median) with 95% confidence interval of 30.9–34.9 years. DNA aneuploidy was determined by flow cytometry analysis in 32 of 85 cervical biopsies (Table 1). The percentage of aneuploid cell clones ranged from 20% in the low-grade lesions (CIN 1) to 32% in the high grade lesions (CIN2/3) and to 80% in the invasive cervical carcinomas. Prevalence of DNA aneuploidy was thus significantly linked to degree of neoplastic progression (P = 0.001, χ² test for trend). However, prevalence of DNA aneuploidy was not influenced by other factors as, for example, women’s age.

Table 1 Prevalence of DNA aneuploidy and transcripts of integrated human papillomavirus type 16 (HPV)-E6/E7 oncoproteins in HPV-16-positive CIN1/2, CIN3, and cervical squamous cell carcinoma (CxCa) tissue samples

Table 2 Frequency table of DNA aneuploidy and integration of viral E6/E7 oncoproteins detected in 85 human papillomavirus (HPV) type 16-positive cervical biopsies (20 CIN1/2, 50 CIN3, and 15 invasive squamous cell carcinomas)

DISCUSSION
Invasive carcinomas of the uterine cervix develop gradually through well-characterized precursor lesions. Usually only very few of the precancerous lesions progress to invasive carcinomas, whereas most lesions either persist or spontaneously regress within a couple of months (12, 13). Thus, the clonal...
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The progression of lesions that express iPOTs additionally supports the concept that iPOTs confer a significantly stronger transforming activity and rapidly push the selection of carcinoma cells. Aneuploidy testing in cervical lesions has been successfully applied to identify high-grade lesions with higher progression tendency. In a refined diagnostic setting, HPV integration analysis can indicate progressing lesions with even higher specificity and might be used as a patient-specific tumor-, metastasis-, and recurrence marker.

REFERENCES


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