Integration of Human Papillomavirus Sequences in Cervical Tumor Cell Lines*

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ABSTRACT

The establishment of three cell lines from keratinizing and nonkeratinizing cervical carcinomas was previously reported. These cell lines were analyzed for growth properties in vitro and in vivo. Metaphases prepared from tissue culture of these cell lines were cytogenetically characterized using GTG-banding and fluorescent in situ hybridization (FISH) using chromosome-specific α-satellite probes. Although the karyotypes of most cells were extremely complex, nonrandom karyotypic abnormalities could be identified. Molecular data had suggested that TC140, derived from a keratinizing cervical tumor, may contain HPV 16 in the episomal state, while TC146, derived from a nonkeratinizing large-cell cervical carcinoma, contained HPV 16 in the integrated state. Therefore, a fluorescent in situ hybridization study was undertaken using biotinylated HPV 16 DNA as a probe on order to confirm and to corroborate the original molecular study, as FISH is the most direct approach for mapping cellular and viral sequences in mammalian chromosomes. The results previously reported in abstract demonstrated the presence of positive hybridization signals on the long arms of the apparent homologs of a human D-group chromosome in cell line TC146. The results of recently completed experiments clearly indicated that while the predominant state of viral existence in the TC140 cell line was apparently episomal, consistent viral integration was found in the TC146 cell line. Furthermore, where viral sequences of HPV 16 integration were observed in cells of TC146, integration was apparently nonrandom. Fluorescent in situ hybridization using various chromosome-specific α-satellite and HPV 16 probes clearly indicated that viral integration occurred nonrandomly and at a specific site on chromosome 13. By chromosome morphometry, the viral integration site was localized to 13q14, also the mapped locus of the retinoblastoma (Rb) tumor suppressor gene.

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Introduction

Cell lines from primary cervical tumors of different histopathological types were established in order to investigate the mechanisms underlying the biological and clinical heterogeneity of cervical tumors.\(^1\)

In a subsequent study,\(^2\) these cell lines were karyotyped. Information obtained complemented previous studies of histopathology and molecular analysis of these three cell lines. Previously available cytogenetic information on solid tumors has been limited because the tumors were often difficult to culture and there were often insufficient metaphases for cytogenetic analysis.

In the present study, our attention is focused narrowly on the integration of the HPV 16 genome into human genetic material. Data obtained in this study complemented molecular data obtained in a previous study.\(^1\)

Materials and Methods

Tissue and Cell Derivation

The origins of the cells were as described in Braun et al.\(^1\) Briefly, TC-140 was derived from a moderately differentiated squamous carcinoma of the cervix with metastases to five of 27 lymph nodes which arose in a 39-year-old woman with a history of condyloma. The TC-146 was derived from an infiltrating, moderately differentiated epidermoid carcinoma of the cervix, predominantly of the large-cell nonkeratinizing type, which arose in a 36-year-old woman with a history of squamous carcinoma-in-situ. This latter patient had metastatic carcinoma in two of three left external iliac nodes.

Cell Harvesting and Slide Preparation

For the conventional and molecular cytogenetic studies, 50 to 55 μL of stock Colcemid* at 10 μg/mL were added to the subconfluent cultures in 10 mL of media for approximately 3 hours prior to harvest. Cells were trypsinized and harvested according to conventional cytogenetic methods, using 0.075 M KCl as the hypotonic agent and three parts methanol to one part glacial acetic acid as the fixative. After repeated rinsing and centrifugation, the pellet suspensions were dropped onto glass slides, which were either air-dried overnight or briefly flamed.

Fluorescent In Situ Hybridization (FISH)

For fluorescent in situ hybridization,\(^3,4,5,6,7,8,9\) the procedure of Pinkel et al.\(^10\) was used with some modifications. Both commercially available viral probes and probes derived from deoxyribonucleic acid (DNA) isolated in one of our laboratories were used. In the latter case, full length human papillomavirus (HPV) 16 DNA was labeled with biotin following standard nick translation procedures\(^11\) and using a commercially available kit. Slides containing metaphase chromosome spreads were pretreated using RNase and 2XSSC, dehydrated in a graded ethanol series, denatured in formamide at 70°C to 71°C and dehydrated again in a cold graded ethanol series.

Alpha-satellite probes were applied to slides and placed in a humidified chamber to hybridize for one hour at 37°C. After one hour, slides were washed in 0.25XSSC for 5 minutes at 72°C. Both human papillomavirus (HPV) 16 prepared by us and commercially available HPV 16/18 probes were denatured for 10 minutes (70°C), chilled quickly, and reannealed for 10 minutes at 37°C. The

\(^*\) Gibco Life Technologies, Inc., Grand Island, NY.
HPV probe was then applied to the slides, covered with glass coverslips and sealed with rubber cement. Slides were then hybridized overnight in a humidified chamber at 37°C.

On the following day, the coverslips were removed and the slides were washed using 50% formamide and 2XSSC at 42°C with agitation. The detection and amplification using fluorescein isothiocyanate (FITC)-labeled avidin and anti-avidin antibodies were done according to manufacturer's instructions.† Metaphases with signals judged to be bright enough were photographed immediately, as the signals were, in general, relatively dim, and the fluorescence quenched rather quickly. Systematic scoring of all cells, protocols for which have been established in this laboratory, was not attempted in this study because of the relatively weak intensity of the signals.

Slides were photographed using a Zeiss epifluorescence photomicroscope using an FITC filter set and Ektachrome ASA 400 color film.

CHROMOSOME MORPHOMETRY

For chromosome morphology, a graphic arts tool (figure 1) was used on metaphase cells projected onto a screen. The procedure for measuring chromosome segments has been extensively described elsewhere.¹²,¹³,¹⁴ Briefly, the position of the fluorescent signal on the propidium iodide stained D-group chromosome was established by determining its fractional length. Normal chromosome bands are then used to determine the location of the signal by multiplying the total absolute length of the chromosome by the fractional distance of the signal from a reference point on the propidium iodide stained chromosome.

Measurements were made by at least two independent observers. Results were also interpreted visually by an American Board of Medical Genetics board certified clinical cytogeneticist.

RESULTS AND DISCUSSION

Previously, the establishment was reported of three cell lines from keratinizing and nonkeratinizing cervical carcinomas, which were analyzed for growth properties in vitro and in vivo. Metaphases prepared from tissue culture of these cell lines were cytogenetically characterized using GTG-banding and FISH using chromosome-specific α-satellite probes. Although the karyotypes of most cells were extremely complex, non-random karyotypic abnormalities could be identified. Molecular data¹ had suggested that TC140, derived from a keratinizing cervical tumor, may contain HPV 16 in the episomal state, while TC146, derived from a nonkeratinizing large-cell cervical carcinoma, contained HPV 16 in the integrated state.

Therefore, a FISH study was undertaken using biotinylated HPV 16 DNA as a probe in order to confirm and to corroborate the original molecular study, as FISH is the most direct approach for mapping cellular and viral sequences on mammalian chromosomes. The results previously reported in abstract demonstrated the presence of positive hybridization signals on the long arms of the apparent homologs of a human D-group chromosome in cell line TC146. The results of recently completed experiments clearly indicated that while the predominant state of viral existence in the TC140 cell line was apparently episomal, consistent viral integration was observed in the TC146 cell line. Furthermore, where viral sequences of HPV 16 integration was observed in cells of TC146, integration was apparently non-

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random, thereby confirming the results of our previous experiments.

Multiple FISH experiments were performed using TC146B cells. Scanning of metaphases and interphases revealed the presence of positive hybridization signals (doublets) in at least a small proportion of cells, although some of the signals on a portion of some slides appear to be nonspecific, probably owing to random local fluctuations of stringency conditions or slight variations in the conditions of fixation. However, it is clear that positive hybridization was observed on the long arms of two apparent homologs of one D-group chromosome in at least a small proportion of cells (figure 2). Fluorescent in situ hybridization on cells derived from TC146A essentially confirmed the presence of hybridization on the acrocentric (D-group) chromosome, although this was found in only two experiments. Results from TC146 are therefore consistent with the molecular finding of HPV 16 DNA being present in TC146 cells in an integrated state.

To identify further the chromosome involved, simultaneous FISH was performed with chromosome 13-, 14- and 15-specific α-satellite probes and the HPV 16 probe under lower stringency conditions. Initial results of this series of experiments excluded chromosome 15 as the acrocentric chromosome with the integration target site. To determine whether the integration site for TC146 was located on chromosome 13 or 14, chromosome 13- and chromosome 14-specific α-satellite probes were utilized. Results of experiments using chromosome 14 α-satellite probe simultaneously with HPV 16 probe revealed the presence of viral integration signals on a D-group chromosome other than 14. By the process of elimination, it was hypothesized that the viral integration occurred nonrandomly on chromosome 13.

To demonstrate unequivocally the integration of viral sequences on chromosome 13, the chromosome 13-specific α-satellite probe was utilized as well as the HPV 16 probe simultaneously in a FISH experiment. The presence of bright positive hybridization signals in the paracentromeric regions of two D-group chromosomes and less intense signals more distally from the centromere clearly indicated that viral integration occurred nonrandomly and at a specific site on chromosome 13. Furthermore, by chromosome morphometry, the viral integration site was localized to 13q14 for cell line TC146 (data not shown).

A search of the literature revealed that Hori et al. had detected HPV 16 DNA sequences integrated near a fragile site via FISH. The site of integration was at 8q24, the locus of the c-myc oncogene. Mincheva et al., on the other hand, found integration of HPV 18 occurring on 8q24 in HeLa cells whereas integration of HPV 16 DNA in SiHa cells was at 13q21–q31. The SiHa cell line is a human cervical squamous carcinoma cell line (ATCC HTB35) that was established from fragments of a primary tissue sam-

**FIGURE 1.** Graphic arts tool used in chromosome morphometry.
ple obtained after surgery from a Japanese patient. Mincheva et al.\textsuperscript{20} using the technique of isotopic (\textsuperscript{3}H-HPV 16 DNA) \textit{in situ} hybridization and the analysis of the distribution of silver grains along the previously banded chromosomes, reported that SiHa cells contain integrated HPV 16 DNA in the region q21–q31 of chromosome 13. In HeLa cells, integration of HPV 18 occurred on 8q24, whereas in CasKi cells at least 11 chromosomal sites of HPV 16 sites of integration were found. On the basis of the findings of varying integration sites in the
host cell DNA Mincheva et al. concluded that integration is random and that "no evidence was obtained for the existence of preferential chromosomal regions for HPV integration." This conclusion was based on the assumption that there is only one consistent chromosomal location for HPV viral integration.

A more realistic hypothesis is that there is more than one viral integration site, depending on the tumor type and what cells within the tumor are involved, as heterogeneity is often found to be a characteristic of these tumors. Data obtained from the present study indeed indicate nonrandom integration in at least a small proportion of cells in the 146 cell line. Whether or not the tumor phenotype observed is a consequence of this particular pattern of integration is an interesting question. The fact that in the HeLa cell line described by Mincheva et al. integration occurred at the protooncogene c-myc and that in our study integration occurred at the mapped locus of the retinoblastoma (Rb) tumor suppressor gene is intriguing. To the best of our knowledge, the present study is the first report of a viral integration site in cervical cancer localized to 13q14, the mapped locus of Rb. Additional data will be required to clarify further the relationship between the disruptions of proto-oncogenes such as c-myc and tumor suppressor genes such as Rb in the multi-step mechanisms leading to carcinogenesis. Pertaining to the localization of the integration site by Mincheva et al. at chromosome 13q21–q31, it should be noted that the distribution of silver grains was not discreet but rather spread out. Consequently, the possibility that their integration site also coincides with ours cannot be ruled out.

Previous molecular data had indicated that TC140 harbored approximately 100 to 200 copies of HPV DNA. Despite repeated attempts involving scanning of approximately 250 metaphases and nearly 5,000 interphase cells, FISH of TC140 cells did not yield consistent positive hybridization signals over any particular chromosome, although hybridization patterns not inconsistent with viral integration were transiently detected in a small subpopulation of cells. In addition, multiple fluorescent signals were observed from time to time. These fluorescent foci, the identities of which cannot be unambiguously ascertained, were often specifically confined to the cell nuclei. The question arose as to whether these positive hybridization spots represent episomal copies of the viral genome or random noise. Informal consultation with world renowned experts at the Gordon Conference in Molecular Cytogenetics during the summer of 1994, however, failed to elicit a satisfactory explanation. To the best of our knowledge, no one had ever demonstrated unambiguously the presence of episomal sequences cytogenetically.

Although the exact role that these HPV sequences played in the genesis of the original cervical tumors is not known, it is not unreasonable to hypothesize that integrations of the viral sequences, similar to structural chromosomal rearrangements such as translocations, may disrupt the function of one or more cellular proto-oncogenes, thus converting it to an oncogene, which eventually leads to cervical cancer. Alternatively, viral integration may disrupt the function of both copies of a tumor suppressor gene. Our finding of viral integration at or near the mapped locus of the Rb gene on 13q14 and also the DBM (disrupted in B-cell malignancy) tumor suppressor gene mapped telomeric to the Rb gene recently is indeed intriguing. The recent localization of the BRCA2 gene on 13q12–13 generated additional excitement about genes located on the long arms of chromosome 13. Further speculation at this point in time, however, is premature.
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