Tetraploidy and chromosomal instability are early events during cervical carcinogenesis

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Introduction

Aneuploidy is the most prevalent genetic change observed in human solid tumors (1), yet the molecular basis responsible for its development during carcinogenesis remains, in most cases, undefined and controversial (2). One hypothesis indicates that mutations in genes that encode for important mitotic proteins can shape the process defined as chromosomal instability, creating an environment where aneuploidy and cancer can develop (3,4). Another hypothesis proposes that aneuploidy is itself the cause of genetic instability and cancer (5,6). While the underlying causes of aneuploidy remain debated, there is reason to believe that polyploidy may constitute an integral step in the evolution of aneuploidy and carcinogenesis (7). There are currently several lines of evidence to suggest that tetraploidy is an important intermediate chromosomal state that plays a major role in the development of aneuploidy in vivo: (i) data from in vitro experiments and computer-generated models demonstrate that aneuploidy may develop through chromosomal loss from a tetraploid intermediate (8,9); (ii) cytogenetic and flow cytometric studies have demonstrated that tetraploidy is present in human solid tumors originating in a variety of tissues (10–15), with aneuploidy arising via chromosomal loss from a tetraploid intermediate being clearly demonstrated in Barrett’s esophagus (16); and (iii) numerical centrosome abnormalities (a genetic anomaly associated with genetic instability (17)) have been shown in some cases to be the by-products of, and not the cause of, tetraploidy (18,19). Together these results suggest that tetraploidy may be a key intermediate common to many human solid tumors, which, primarily through subsequent chromosomal loss, can develop into more advanced aneuploid tumors.

Numerical chromosomal aberrations such as aneuploidy and tetraploidy have been reported in women diagnosed with precancerous and cancerous cervical lesions (20–28). Because tetraploid cervical cells are often observed in precancerous lesions whereas aneuploid cells are frequently observed in the cancerous lesions, several investigators have hypothesized that a sequential pattern of chromosomal aberrations occurs during cervical carcinogenesis, where aneuploidy develops through chromosomal loss from a tetraploid intermediate (29–31). However, these observations have been based on data obtained from small numbers of samples, with relatively few cells analyzed for each sample.

The aim of this study was to more fully investigate the hypothesis that aneuploidy develops through chromosomal loss from a tetraploid intermediate. Our studies were performed using DNA probes for Chromosomes 3 and 17, chromosomes previously reported to exhibit non-random chromosome alterations in cervical cancer cells (28,32,33), to study the numerical chromosomal aberrations during cervical carcinogenesis. In addition, pancentromeric probes were employed to monitor chromosomal loss and breakage occurring as micronuclei. The results indicate that tetraploidy and

Abbreviations: ASCUS, atypical squamous cells of undetermined significance; DAPI, 4',6-Diamidino-2-phenylindole; FISH: fluorescence in situ hybridization; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; SSC, saline–sodium citrate.
chromosomal instability are related events occurring during the early stages of cervical carcinogenesis that predispose cervical cells to the formation of aneuploidy frequently involving the loss of Chromosome 17.

Materials and methods

Cervical cell collection and preparation

Cervical cells were obtained from 143 different women attending the Mexican National Cancer Institute in Mexico City, Mexico for gynecological examination. Women were informed of the study’s goals and those that agreed to participate signed a consent form and completed a questionnaire. Pap smears were prepared and analyzed according to the Bethesda classification system by cytopathologists at the Mexican National Cancer Institute, and were diagnosed as Normal ($n = 39$), Inflammatory ($n = 48$), atypical squamous cells of undetermined significance (ASCUS) ($n = 6$), low-grade squamous intraepithelial lesions (LSIL) ($n = 9$) or as high-grade squamous intraepithelial lesions (HSIL) ($n = 41$). A cell suspension of residual cervical cells was obtained by vortexing the cytobrush in phosphate buffered saline and was treated with 10% Mucomyst (Mead Johnson; Evansville, IN) following the preparation of the original Pap smear. The cells were removed from the phosphate buffered saline (PBS) mixture and fixed in a methanol:acetic acid mixture (3:1). Several pathologists analyzed the Pap smears collected from the participants of this study and only in those cases where there was agreement with the diagnostic classification were the preserved cervical cells shipped to the University of California, Riverside for the cytogenetic analysis. Approval from the Mexican National Cancer Institute and the UC Riverside Institutional Review Board (IRB) was obtained prior to the beginning of the study.

Cytogenetic analysis, fluorescence in situ hybridization

Cervical cells were dropped onto clean glass microscope slides and stored at $-20^\circ$C in a nitrogen atmosphere. The slides were processed for dual-probe fluorescence in situ hybridization (FISH) by treating the slides with 2% paraformaldehyde at 4°C for 20 s and briefly rinsing them twice in 2 × SSC (saline–sodium citrate) at room temperature. The cellular DNA on the slides was denatured using 70% formamide/2 × SSC at 68.5°C for 2 min and 45 s, dehydrated in an ethanol series (70, 85 and 100%) for 2 min each and dried under nitrogen gas. Slides were subsequently treated with 5 μg/ml proteinase K in 2 × SSC at 37°C for 13 minutes. Following proteinase K treatment, the slides were briefly rinsed twice in 2 × SSC at room temperature and processed through another ethanol series (70, 85 and 100%) for 1 min each and allowed to air dry. Probes specific for the α-satellite regions of Chromosomes 3 (D3Z1) and 17 (D17Z1) were generated using a protocol described previously (34). The hybridization mixture containing the α-satellite Chromosomes 3 and 17 centromeric probes was heated at 70°C for 5 min, applied to the slide, and incubated overnight in a humidified chamber at 37°C. Non-specific hybridizations were removed by washing the slides three times in 60% formamide/2 × SSC at 43°C for 5 min. The probes were detected using Alexa 555-conjugated avidin (Chromosome 3) and fluorescinated anti-digoxigenin IgG (Chromosome 17), while DAPI (0.25 μg/ml) was used to counterstain the cell nuclei. All the slides were coded and analyzed in a blind fashion at 1250× using a fluorescent Nikon microscope with a triple-band-pass filter (excitation at 375–395, 515–555 and 610–660 nm, respectively; Chroma Technologies, Rockingham, CT). One thousand epithelial cervical cells were analyzed per sample for numerical chromosomal aberrations. Scoring criteria for the dual-probe FISH have been described previously (35). A cell was considered tetraploid if it was tetrasomic for both Chromosomes 3 and 17 (Figure 1A). Previous studies from our laboratory have shown that the simultaneous measurement of the copy number of two chromosomes is an efficient measure of ploidy level (very similar frequencies of tetraploidy and hyperdiploidy aneuploidy were seen when samples were evaluated using probes for Chromosomes 3 and 17 or 1 and 9) in cervical cells diagnosed as LSIL (36). Following a similar criteria, a cell was identified to be hyperdiploid-aneuploid if it had a chromosome (3 and 17) complement that was greater than diploid but differed from a triploid or tetraploid cell, whereas a cell was determined to be hypo-diploid if any chromosome complement was less than diploid. Near-tetraploid aneuploidy was defined as a cell that was trisomic for one chromosome but tetrasomic for the other [e.g. four copies of Chromosome 3 and three copies of Chromosome 17 (Figure 1B)]. While hypo-diploid cervical cells were occasionally observed, they can often result from signal overlap or inefficient hybridization as well as true chromosomal imbalances (37) and, as such, were not included in the final analyses.

Fig. 1. Examples of (A) tetraploid cervical cell, (B) near-tetraploid aneuploid (tetrasomy 3 and trisomy 17) cervical cell and (C) cervical cell containing a centromere-positive micronucleus. See online supplementary material for a color version of this figure.

Cytogenetic analysis of micronuclei

Cervical cells from 74 of the donors who had a sufficient number of cells were cytopspun or dropped onto clean glass slides and stored at $-20^\circ$C in a nitrogen atmosphere. The slides were processed for the micronucleus assay using the same protocol as was used for the dual-probe FISH assay except that a pancentromeric probe (38) was used. Non-specific hybridizations were removed by washing the slides three times in 2 × SSC at 43°C for 5 min. The probe was detected using a fluorescinated anti-digoxigenin IgG, while DAPI (1 μg/ml) was used to counterstain the cell nuclei. Two thousand cervical epithelial cells were analyzed per sample for the presence of micronuclei. Micronuclei labeling with a centromeric probe were defined as being formed through chromosomal loss (Figure 1C), whereas micronuclei not being formed contained the centromeric sequences were classified as being formed from chromosomal breakage. While it is possible that micronuclei labeling with a centromeric probe also contain chromosomal fragments, this is highly unlikely because of the low frequency of chromosome loss and breakage observed in these cells (Table II). Previous work has demonstrated that the use of a centromeric DNA probe is an efficient method to distinguish between micronuclei that have been formed through chromosomal loss and breakage (38).

Statistical analysis

A Kruskal–Wallis test was used to analyze the numerical chromosomal aberration data obtained through the FISH analysis. Following a significant Kruskal–Wallis result ($P$-value $<0.05$), a post-hoc Mann–Whitney U-test was used to compare the tetraploid and aneuploid frequencies of each of the diagnostic categories with the Normal diagnostic category. Analysis of variance was used to determine whether there were significant increases in the number of micronuclei formed through chromosomal loss and breakage compared with controls. A post-hoc Fisher’s protected least significant difference
test was used to identify groups that differed significantly from the control group. Regression analysis was used to determine whether there were correlations between the frequency of micronucleated cervical cells and the frequencies of hyperdiploid-aneuploid, near-tetraploid aneuploid, and tetraploid cervical cells, as well as to determine whether a correlation existed between the frequencies of tetraploid and near-tetraploid aneuploid cervical cells. One clear outlier was excluded in the regression analyses of the aneuploid and near-tetraploid aneuploid data. All analyses were conducted using the Statview statistical software (Cary, NC). A two-tailed Fisher’s exact test was used to identify whether Chromosome 3 or 17 was preferentially lost or gained from near-tetraploid aneuploid cervical cells. P-values of <0.05 were considered significant for all analyses.

Results

We analyzed 1000 cervical cells from each of 143 different women, separated into different diagnostic categories for numerical chromosomal aberrations using dual-probe FISH for the simultaneous analysis of Chromosomes 3 and 17. The frequency of tetrasomic and aneusemic cells were significantly elevated over the Normal category for the Inflammatory, ASCUS, LSIL and HSIL categories (Table I). Cervical cells tetrasomic for Chromosomes 3 and 17 were often observed in the absence of aneusomy (34/143 cases, 23.8%), whereas aneusomy rarely occurred in the absence of tetrasomy (6/143 cases, 4.2%). In addition, a majority of the cases that contained elevated levels of aneusomy were identified as having near-tetraploid aneusomic cervical cells (26/35 cases, 74.3%). A significant association between the number of tetrasomic and near-tetraploid aneusomic cells was also identified (P = 0.0019; Figure 2).

Cervical cells were analyzed for chromosomal instability in the form of micronuclei to determine whether chromosome mal-segregation could be detected during the hypothesized tetraploid-to-aneuploid transformation period. Micronucleus frequencies were evaluated in 2000 cervical cells from 74 of the women and a pancentromeric DNA probe was used to discriminate between micronuclei that had formed through chromosomal loss and breakage. Significant increases in cervical cells exhibiting both chromosome loss (centromere-positive micronuclei) and breakage (centromere-lacking micronuclei) were seen in both the LSIL and HSIL categories (Table II). Significant positive correlations (P-values of <0.0001) were observed between tetrasomy and centromere-negative (R² = 0.56), centromere-positive (R² = 0.44) and total (R² = 0.69) micronuclei (Figure 3A–C). Significant positive correlations (P-values of <0.0001) were also observed between aneusomy and centromere-negative (R² = 0.34), centromere-positive (R² = 0.24) and total (R² = 0.38) micronuclei (Figure 4A–C).

A distinct pattern of aneusomy became apparent during FISH analyses, in which cervical cells predominantly exhibited trisomy 17 and tetrasomy 3 (Figure 1B). Analysis of the HSIL category demonstrated that majority of the cases (28/41) exhibited this pattern and the incidence was significantly different from that seen in the Normal category (3/39, P < 0.0001; Figure 5). Interestingly, no preferential loss of Chromosome 3 was observed in these samples. To determine whether these results were artifacts due to our FISH scoring criteria or the particular probe utilized for Chromosome 17, we analyzed the data to determine whether the preferential loss of Chromosome 17 occurred in the hypo-diploid aneuploid cells (cells exhibiting two copies of Chromosome 3 and one copy of Chromosome 17). While some monosomy 17 was observed in

Table I. Frequencies of tetrasomy and aneusomy in the cervical cells of women

<table>
<thead>
<tr>
<th>Category of Women</th>
<th>N</th>
<th>Tetrasomy</th>
<th>Aneusomy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median IQR</td>
<td>P-value</td>
</tr>
<tr>
<td>Normal</td>
<td>39</td>
<td>0–2</td>
<td>—</td>
</tr>
<tr>
<td>Inflammatory</td>
<td>48</td>
<td>1–8.5</td>
<td>0.001</td>
</tr>
<tr>
<td>ASCUS</td>
<td>6</td>
<td>4–28</td>
<td>0.0006</td>
</tr>
<tr>
<td>LSIL</td>
<td>9</td>
<td>2.8–48.5</td>
<td>0.0011</td>
</tr>
<tr>
<td>HSIL</td>
<td>41</td>
<td>4–49.8</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Both tetraploidy and aneuploidy were significantly elevated for all categories when compared with the Normals.

*Statistically significant, Mann–Whitney U-test.

**IQR** is the inter-quartile range.

Table II. Frequencies of micronucleated cells formed from both chromosomal loss and breakage in the cervical cells of women

<table>
<thead>
<tr>
<th>Category of Women</th>
<th>N</th>
<th>Total</th>
<th>Chromosomal loss</th>
<th>Chromosomal breakage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>P-value</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>24</td>
<td>5.8</td>
<td>3.1</td>
<td>—</td>
</tr>
<tr>
<td>Inflammatory</td>
<td>20</td>
<td>8.5</td>
<td>5.5</td>
<td>0.25</td>
</tr>
<tr>
<td>ASCUS</td>
<td>1</td>
<td>14</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>LSIL</td>
<td>6</td>
<td>15.5</td>
<td>15.1</td>
<td>0.0074</td>
</tr>
<tr>
<td>HSIL</td>
<td>23</td>
<td>13.4</td>
<td>9.8</td>
<td>0.0011</td>
</tr>
</tbody>
</table>

Micronucleus frequencies were derived from the analysis of 2000 cells per individual.

*Statistically significant, with a P-value ≤ 0.05 by ANOVA followed by the Fishers protected least significant difference test.
and breakage (aneusomy and micronucleated cells formed through both chromosomal loss and breakage) were identified to be significantly elevated in the LSIL and HSIL diagnostic categories (Table II). Furthermore, as FISH data for Chromosomes 3 and 17 were available for these samples, we were able to correlate a biomarker of chromosomal instability with both tetrasomy and aneusomy. Micronuclei (total as well as those from chromosomal loss and breakage) were identified to be significantly unstable phenotype prone to undergo more chromosomal changes. Interestingly, the correlations between aneusomy and micronucleated cells formed through both chromosomal loss and breakage (R²-value of 0.685; regression equation: Y = 6.751 + 0.169X). All correlations are statistically significant (P-value <0.0001).

**Discussion**

The initial objective of this study was to determine the role of tetraploidy in the formation of aneuploidy and ascertain the occurrences of these changes during the early stages of cervical carcinogenesis. The patterns identified in this molecular cytogenetic study indicate that tetraploidy is an early and common event during cervical carcinogenesis that precedes the development of aneuploidy. The presence of significantly elevated levels of tetrasomy and aneusomy during the various stages of cervical carcinogenesis are consistent with previous findings (20–28) and indicate that chromosomal aberrations are common for this type of epithelial tumor. However, the identification of unique patterns in which tetrasomy occurs either by itself or in combination with aneusomy, whereas aneusomy is largely dependent upon the presence of tetrasomy, combined with the observation that near-tetraploid aneusomic cells strongly correlate with tetrasomy (Figure 2), provide support for previous findings (29–31) indicating that these chromosomal alterations occurred sequentially, where tetraploidy precedes the development of aneuploidy.

Additional analyses were also performed to monitor chromosomal instability in the form of micronucleated cervical cells. Previous studies have detected elevated frequencies of micronuclei in women with precancerous and cancerous cervical lesions (39–41) but did not identify how the micronuclei were formed. Using a pancentromeric DNA probe, we were able to detect the presence or absence of centromeric sequences in the micronucleus and determine its origin. Using this technique, micronuclei forming through both chromosomal loss and breakage were identified to be significantly elevated in the LSIL and HSIL diagnostic categories (Table II). Furthermore, as FISH data for Chromosomes 3 and 17 were available for these samples, we were able to correlate a biomarker of chromosomal instability with both tetrasomy and aneusomy. Micronuclei (total as well as those formed through either chromosomal loss or breakage) were highly correlated with tetrasomy (Figure 3). Because tetrasomy is significantly linked to a biomarker of chromosomal instability, it suggests that tetraploidy is a chromosomally unstable phenotype prone to undergo more chromosomal changes. Interestingly, the correlations between aneusomy
and numerical aberrations targeting Chromosome 17 are often the observed non-random loss of this chromosome. Structural BRCA1 and p53 carcinogenesis. The genes for a variety of tumor suppressor some 17 is likely to be a relatively early event in cervical diagnosis as LSIL and HSIL, whereas they were previously distinguished characteristic of the current study, however, is that dual-probe FISH to simultaneously analyze Chromosomes 3 and 17 was an efficient indicator of the ploidy level (both tetraploidy and aneuploidy, as estimated on the same samples using dual-probe FISH for Chromosomes 1 and 9) in cervical cells diagnosed as LSIL (36). Therefore, in spite of the aforementioned caveat, the dual-probe results from the current study indicate that tetraploidy is an early and chromosomally unstable intermediate that precedes the development of aneuploidy during cervical carcinogenesis.

Results from the current molecular cytogenetic study suggest that chromosomal loss frequently occurs from a chromosomally unstable tetraploid phenotype, leading to the formation of aneuploid lesions during cervical carcinogenesis. One question that these data raise is whether the observed chromosomal instability in the tetraploid cells is due to their tetraploid nature or due to another factor. Epidemiological data clearly indicate that persistent human papilloma virus (HPV) infection is the main etiological agent associated with the development of cervical cancer (47,48). The correlation between cervical cancer and infection with HPV 16 and 18 is sufficiently strong that the International Agency for the Research on Cancer (49) has classified them as known human carcinogens. There is also evidence to suggest that HPV infection may be responsible for the underlying chromosomal instability that can create a cellular environment where tetraploid cells can develop and proliferate. The E6 and E7 viral oncoproteins are capable of binding to and abrogating the function of p53, p21 and pRb, the key cellular proteins that strictly regulate the cell cycle and DNA synthesis (50,51). Tetraploidy has been observed to develop in cells that are lacking these important cellular proteins (52–55). The numerical chromosomal aberrations observed during cervical carcinogenesis are therefore the probable result of infection by cancer-associated types of HPV and originate from the virus’s ability to disrupt nuclear and cellular division. Data from several studies have reported that the presence of tetraploid cervical epithelial cells was highly correlated with HPV infection (20,21,36,56) and can be induced through the expression of the E6 and E7 proteins in vitro (57,58). Furthermore, numerous structural chromosomal aberrations have been observed during cervical carcinogenesis (59–62) and may be the result of HPV integration into the cellular genome during disease progression (63,64). Indeed, recent observations indicate that the integration of HPV results in chromosomal instability in vitro (65), with differences in chromosomal instability being observed between episomal and integrated HPV infections (66). In addition, anaphase bridging and the loss of flanking cellular DNA have been observed to occur during and following viral integration (67–69) and could potentially contribute to the formation of the fragment-containing micronuclei that were observed in this study (70).
Because the development of numerical chromosomal aberrations follows a sequential pattern during cervical carcinogenesis, where tetraploidy often precedes the development of aneuploidy, a woman with elevated levels of tetraploidy may be at an increased risk of developing an advanced cervical lesion. Thus the identification of elevated levels of tetraploidy and/or aneuploidy in cervical cells could serve as a molecular diagnostic tool to be used in conjunction with the ASCUS Pap smear. There are ~2.5 million women each year in the USA who are diagnosed with an ASCUS Pap smear (71), many of whom undergo further costly, stressful, and more invasive procedures to ensure that a precancerous or cancerous cervical lesion is not present. The identification of a molecular biomarker of persistent HPV infection in women who have been diagnosed with an ASCUS Pap smear could be a valuable tool in the proper management of these women. Parallel studies in our laboratory have shown that a significant proportion of women diagnosed as ASCUS and HPV-positive exhibit elevated levels of tetraxploid cervical cells (36), indicating that these chromosomal aberrations are early events that can be detected prior to the onset of more serious cervical lesions.

In summary, these results provide evidence that both chromosomal loss and breakage occur during cervical cancer development and that micronuclei, an established biomarker of genetic instability, is highly correlated with the presence of tetraploidy in the progressing cervical lesions. Results from this study provide evidence that numerical chromosomal aberrations occur through a sequential pattern, where tetraploidy often precedes the development of aneuploidy, and that Chromosome 17 is often preferentially lost leading to near-tetraploid cervical lesions. Lastly, the patterns identified from these cytogenetic studies indicate that tetraploidy is a transient and chromosomally unstable intermediate in the development of aneuploid cervical lesions.

Supplementary material
Supplementary material can be found at http://www.carcin.oxfordjournals.org.

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Conflict of Interest Statement: None declared.

References
Chromosomal instability during cervical carcinogenesis


