Genomic integration of oncogenic HPV and gain of the human telomerase gene TERC at 3q26 are strongly associated events in the progression of uterine cervical dysplasia to invasive cancer

AHN Hopman,1* W Theelen,1† PPH Hommelberg,1† MAF Kamps,1 CS Herrington,2 LE Morrison,3 EJMJ Speel,1 F Smets4 and FCS Ramaekers1

1Department of Molecular Cell Biology, Research Institute Growth & Development (GROW), University of Maastricht, The Netherlands
2Bute Medical School, University of St Andrews, St Andrews KY16 9TS, UK
3Abbott Molecular Inc, IL, USA
4Department of Pathology, Foundation of Collaborating Hospitals of Eastern Groningen (SSZOG), Winschoten, The Netherlands

*Correspondence to: AHN Hopman, Phd, Department of Molecular Cell Biology (Box 17), University of Maastricht, PO Box 616, 6200 MD Maastricht, The Netherlands. E-mail: Hopman@molcelb.unimaas.nl

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†These authors contributed equally to this study.

Abstract

Recently proposed events associated with the progression of cervical intraepithelial neoplasia (CIN) 2/3 to cervical carcinoma include integration of human papillomavirus (HPV) into the host genome, genomic instability, and an increase in chromosome 3q copy number. In particular, the gene coding for the RNA component of telomerase (TERC) at 3q26 has been implicated as a possible candidate gene. Since it is not known to date how these events are temporally related during cervical carcinogenesis, the aim of the present study was to assess the correlation between TERC gene copy number and the physical status of HPV during progression in cervical neoplasia. Solitary precursor lesions of the uterine cervix (CIN 2/3, n = 17), lesions associated with a micro-invasive carcinoma (CIN 3&mCA, n = 13), and advanced invasive carcinomas (invCA, n = 7) were analysed by fluorescence in situ hybridization (FISH) to determine the physical status of the virus and TERC gene copy number. The TERC gene was increasingly gained with progression of CIN 2/3 (3 of 17) through CIN 3&mCA (7 of 13) to invCA (5 of 7). In the lesions exhibiting gain of TERC, the virus was predominantly integrated. This was seen in eight of ten diploid lesions, indicating that these events can occur prior to aneuploidization and are strongly associated with the progression of CIN 3 to mCA and invCA (p < 0.001). With progression to carcinoma, a number of these lesions show polyploidization, resulting in aneuploidy and high TERC gene copy numbers. In conclusion, genomic integration of oncogenic HPV and gain of the human telomerase gene TERC appear to be important associated genetic events in the progression of uterine cervical dysplasia to invasive cancer.

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Keywords: uterine cervix; dysplasia; SIL; CIN; HPV 16/18; HPV integration; chromosomal aberrations; telomerase amplification

Introduction

Uterine cervical cancer is the second most common gynaecological malignancy in the world in both incidence and mortality. Several premalignant stages can be distinguished in the development of invasive carcinoma, including cervical intraepithelial neoplasia grades 1–3 (CIN 1–3). The vast majority of CIN 1 lesions regress spontaneously and only a few lesions persist or progress to CIN 2/3 and invasive carcinoma (invCA) [1–3]. Progression has been associated independently with three different genetic events, ie integration of high-risk papillomavirus into the cellular genome [4,5], accumulation of numerical chromosome aberrations [6–9], and development of genomic instability with a consistent gain of chromosome arm 3q [10–13]. Infection with oncogenic human papillomaviruses (HPVs) is considered an initiating factor in the carcinogenesis of the uterine cervix [1,3]. Although 95% of patients with precancerous lesions harbour oncogenic HPV, only a small fraction of these eventually progresses to invCA [2]. Therefore HPV infection alone is considered insufficient for malignant conversion. There is consensus that integration is common in most cases of invCA (high expression of E6/E7) and that integration is
uncommon or absent in CIN 1 [4,14–16]. However, the precise percentage of CIN 2/3 lesions in which HPV is integrated is controversial [8,15,17].

Chromosomal aberrations in CIN 1 are only reported in lesions containing episomal HPV, mainly comprising tetrasonies for a number of chromosomes. [6,18] Aneuysomies are a frequent phenomenon in CIN 2/3 and invCA. For this reason, several authors have proposed that progression from CIN 2/3 to carcinoma correlates with increasing chromosomal aneuysomy, loss of heterogeneity (LOH), and genetic instability [3,6–8,19,20]. Many different patterns of numerical abnormality have been reported in cervical carcinoma, but the most recurrent structural chromosomal aberration in cervical cancer is 3q gain. This aberration was found to mark the transition from high-grade pre-malignant lesions to invCA and the smallest consensus region of 3q amplification in cervical cancer was mapped to chromosomal bands 3q26–27, suggesting that this region contains genes that are involved in cervical carcinogenesis [21–24]. In this respect, the TERC gene, which encodes the telomerase RNA template, which is one of the two essential components of the telomerase–enzyme complex, has been demonstrated to be of particular importance for cervical cancer [25–28]. Expression of the TERC gene and viral oncopgenes increases significantly with histopathological severity of the lesion [29]. Furthermore, viral proteins are involved in the transcriptional regulation of the other component of the complex, the telomerase gene [30]. Recently, Heselmeyer-Haddad et al reported that targeting the TERC gene on chromosome 3q and establishing its copy number in routinely prepared cytological material by means of FISH could serve as a test to determine the progressive potential of individual CIN 2/3 lesions [11,12]. In a retrospective study, these authors showed that detection of gain of TERC in cytologically low-grade lesions and normal Pap smears may predict progression from Pap I–II to Pap IV. However, the physical status of HPV and histological analysis of 3q gain were not assessed in these studies.

The aim of the present study was to analyse the relationships between the physical status of oncogenic HPV, chromosome instability, and gain of TERC copy number simultaneously.

Materials and methods

Tissue material

Formalin-fixed, paraffin-embedded endocervical and ectocervical biopsies, diathermy loop excisions, and cold knife cervical conisation samples were selected from the files of the Department of Pathology (Foudation of Collaborating Hospitals of Eastern Groningen), Winschoten, The Netherlands and the Department of Pathology, Royal Liverpool University Hospital, Liverpool, UK. This study was carried out in accordance with local ethical guidelines. The cervical biopsies were classified independently according to WHO criteria. Thirty-seven cases of high-grade cervical intraepithelial neoplasia (CIN) 2/3 and invCA were analysed and comprised solitary CIN 2/3 lesions (n = 17), CIN 3 associated with a micro-invasive carcinoma (n = 13, CIN 3&mCA), and invCA (stage IB and higher, n = 7). In the cases of CIN 3&mCA, only the CIN 3 component adjacent to the mCA was analysed.

Fluorescence in situ hybridization (FISH)

FISH analysis was performed on 4 µm thick paraffin tissue sections as described previously in detail [31–33]. The directly labelled probes for chromosome 3 (3c) (Vysis SpectrumGreen™ CEP3), chromosome 7 (7c) (Vysis SpectrumAqua™ CEPT), and the TERC region on 3q26.3 (SpectrumOrange™ TERC) were hybridized in a mixture (Vysis, Abbott Molecular, Des Plaines, IL, USA). The digoxigenin-labelled HPV 16/18 (mixture) was obtained from PanPath (Amsterdam, The Netherlands). Two separate pre-treatment procedures were used: the mild and the harsh pretreatment methods [32]. The mild pretreatment targeted episomal and integrated DNA as well as viral RNA. The harsh protocol, which extracts cytoplasmic and nuclear proteins as well as RNA and episomal DNA, was used to assess integrated HPV and chromosomal targets (7c, 3c, TERC). The probe and target DNA were denatured simultaneously and hybridized overnight at 37°C. After stringent post-hybridization washes, the specimens hybridized with the probe mixture (7c, 3c, TERC) were embedded in an anti-fade solution containing 4',6-diamidino-2-phenylindole (DAPI, Sigma) to counterstain the DNA. The digoxigenin-labelled HPV probes were visualized using the tyramide signal amplification (TSA) procedure using rhodamine (TRITC)-labelled tyramide [33–35].

Evaluation of FISH signals

HPV probes

Evaluation of the HPV signals was performed according to the criteria described previously [31,32,36]. In brief, the patterns were (1) a diffuse pattern representing episomal HPV that correlates with viral replication; (2) a punctate pattern consisting of one or a few discrete signal(s) in the nucleus, indicating HPV integration into the cellular genome; and (3) a mixed pattern with separate areas containing only integrated or episomal copies, and areas where the integrated virus was hidden in episomal HPV copies.

Chromosome probes

Hybridizations on lymphocytes, stromal cells, and/or endothelial cells were used as internal controls. The copy number for chromosome 7 was used as a
control for the overall DNA ploidy of cells. [11,12] The maximum number of signals per nucleus was determined and used as an indicator of copy number when more than 10–20% of the nuclei exhibited this number of FISH signals. In this way, the lesions could be classified as diploid, tetraploid or aneuploid [8,37]. In a subset of lesions, the number of dots for 7c, 3c, and TERC was counted in 100–200 nuclei to verify copy number estimations.

### Results

#### TERC gain in cervical (pre)neoplasia

The FISH results in the three groups of lesions are summarized in Figure 1 and Table 1. Representative FISH images and signal distributions are illustrated in Figures 2 and 3.

Six of 17 lesions from the group of solitary CIN 2/3 lesions showed disomy for all chromosomal targets, suggesting a diploid DNA content (Figures 2B and 3A). The other 11 lesions showed numerical aberrations for at least one of the targets. In eight of these lesions, copy numbers for all targets were the same; six of these were tetrasomic (cases 10–15; Figures 2C and 3C) and two showed an aneusomy (cases 16 and 17). Gain of TERC was found in the remaining three cases (cases 7–9; Figures 2E and 3B), two of which contained cells with a diploid DNA content.

In the 13 CIN 3/mCA lesions, only one case showed disomy for all chromosomal targets (case 18), while numerical aberrations were found in the remaining samples.

In five lesions, an aneusomy was detected for chromosomes 3, 7, and TERC, but the copy numbers were balanced (cases 26–30; Figures 2F and 3D). Gain of TERC was found in the remaining seven lesions (cases 19–25), with a typical trisomy for TERC in cells with a DNA diploid background in four cases (cases 19–22; Figure 2E). The lesions with a very high copy number for the centromeres of chromosomes 3 and 7 frequently showed extreme heterogeneity of nuclear size, hampering the estimation of true chromosome copy numbers, due to nuclear truncation. Gain of TERC was, however, easily determined when analysing individual nuclei.

In six of seven invasive carcinomas, numerical alterations were observed for the three DNA targets: five of these showed a gain of TERC (Figures 2G, 2I, and 3E).

Comparison of the FISH results between the three groups showed that apart from diploid samples, tetraploid lesions were predominantly found in the solitary CIN 2/3 group, while CIN 3&mCA and invCA lesions exhibited DNA aneuploidy more often.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** FISH copy number distributions for the chromosome 7 centromere (□), the chromosome 3 centromere (×), and TERC (○), and the physical status of HPV as determined by FISH in CIN 2/3, CIN 3 adjacent to micro-invasive carcinomas (CIN 3&mCA), and invasive carcinomas (invCA). The highest copy numbers per nucleus are indicated. Equal copy numbers for all targets are indicated by ■.

### Table 1. Distribution of DNA ploidy and TERC gene copy number in CIN 2/3, CIN 3&mCA, and invCA

<table>
<thead>
<tr>
<th></th>
<th>Estimated overall DNA ploidy n (%)</th>
<th>Gain of TERC n of total (%)</th>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>Diploid</td>
</tr>
<tr>
<td>CIN 2/3</td>
<td>17</td>
<td>8 (47)</td>
</tr>
<tr>
<td>CIN 3&amp;mCA</td>
<td>13</td>
<td>8 (61)</td>
</tr>
<tr>
<td>invCA</td>
<td>7</td>
<td>2 (29)</td>
</tr>
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</table>

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Figure 2. (A, D, H) Representative examples of FISH analyses for HPV 16/18. (A) A diffuse pattern indicating replicating, episomal HPV (in red) (case 4, CIN 2/3). (D) Combined diffuse and punctate signals indicating episomal and integrated HPV (D, case 7, CIN 2/3). (H) Nuclei showing multiple HPV integration sites (red). (B, C, E–G, I) Representative examples of FISH analysis using a probe mixture for the chromosome 3 and 7 centromeres, and the TERC gene, on paraffin-embedded tissue sections from patients diagnosed with cervical (pre)neoplasia. Double-target imaging of chromosome 3 (green) and TERC (red) shows (B) disomy for both targets (case 4), (C) tetrasomy for both targets (case 11, CIN 2/3), (E) disomy for chromosome 3 and trisomy for TERC (case 7, CIN 2/3), (F) aneusomy for chromosome 3 and TERC (case 28, CIN 3&mCA), and (G) gain of TERC as compared to chromosome 3 (case 34, invCA). Nuclear counter-staining in FISH with DAPI (blue). FISH triple-target imaging of chromosome 3 (green), TERC (red), and chromosome 7 (blue) (I) shows gain (amplification) of TERC as compared to chromosome 3 and chromosome 7 (case 36, invCA). Note that fewer than the indicated signals may be visible per cell in these images due to truncation of the nuclei upon sectioning of the specimen, and signals being below or above the focal plane at which the image was recorded.

These cases were also frequently aberrant for TERC. The difference in copy number aberrations for TERC (excluding tetraploidy) correlated significantly ($p < 0.001$) with solitary CIN 2/3, CIN 3 adjacent to mCA, and invCA. Gain of TERC also correlated significantly with these three classes of lesion ($p < 0.01$). Comparison of solitary CIN 2/3 lesions with CIN 3&mCA and invCA showed that gain of TERC was observed in two diploid solitary lesions and eight diploid mCA/invCA.
Figure 3. FISH spot frequency distributions for the chromosome 3 centromere and TERC gene on tissue sections from patients diagnosed with cervical (pre)neoplasia. (A) Case 4 (CIN II/III); (B) case 7 (CIN 2/3); (C) case 11 (CIN 2/3); (D) case 28 (CIN 3&mCA); (E) case 36 (invCA). Black bars indicate nuclei with gain of TERC.

Table 2. Distribution of HPV integration and TERC gain in DNA diploid, tetraploid, and aneuploid lesions

<table>
<thead>
<tr>
<th>n(HPV+)</th>
<th>Integrated HPV n of total (%)</th>
<th>TERC gain in lesions with HPV int n of total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CIN 2/3</td>
<td>CIN 3&amp;mCA</td>
</tr>
<tr>
<td>Diploid</td>
<td>18 (16)</td>
<td>2/6 (33)</td>
</tr>
<tr>
<td>Tetraploid</td>
<td>8 (7)</td>
<td>0/6</td>
</tr>
<tr>
<td>Aneuploid</td>
<td>11 (11)</td>
<td>1/3 (33)</td>
</tr>
</tbody>
</table>

int = integrated.

Physical status of HPV

The results of the FISH HPV detection studies are summarized in Figure 1 and typical examples are depicted in Figure 2. Both protocols were applied to assess episomal and integrated HPV. Twelve of 15 HPV-positive solitary CIN 2/3 lesions showed an exclusively episomal pattern (see Figure 2A), while viral integration was recognized in three cases. Case 7 contained both episomal and integrated virus, with regions exhibiting integrated virus adjacent to regions containing episomal HPV (Figure 2D). An exclusively episomal pattern was seen in only two of 13 CIN 3&mCA lesions, while the other cases showed an integrated pattern. In five lesions, cells with integrated HPV also exhibited episomal viral copies, as determined by comparing the FISH patterns obtained after application of the mild protocol and harsh protocol, and taking into account the intensity of the different hybridization spots. In the invCA group, six of seven HPV-positive lesions showed an integrated HPV pattern: two of these also showed an episomal pattern. In two of these lesions, we noticed multiple copies of integrated virus (see Figure 2H). In all the other lesions with integrated virus, one single HPV integration spot was observed per nucleus.

Correlation of TERC gain with the physical status of HPV

Figure 1 and Table 2 summarize the detected TERC gain and the physical status of HPV. It is clear that the
Genomic integration of oncogenic HPV and gain of the human telomerase gene TERC

Discussion

This study constitutes a comprehensive evaluation of solitary CIN 2/3 lesions, CIN 2/3 lesions with adjacent (micro)invasive carcinoma, and established invasive lesions with the goal of investigating the relationship between gain of TERC at 3q26 and the physical status of HPV in premalignant lesions. Gain of 3q26 and genomic HPV integration correlate strongly with invasive cervical carcinoma but the reported frequencies of genomic HPV integration in premalignant lesions are inconsistent [1,5,8,15,17]. Moreover, the association between the two events in individual patients has not been previously investigated. In the present study, we demonstrate that HPV genomic integration and gain of the telomerase gene TERC (3q26) are strongly associated events in the transition of uterine cervical dysplasia to invasive carcinoma. In this respect, the transition of episomal to integrated oncogenic virus is a risk factor, which we now show to be associated with TERC gain. This indicates that TERC gain and HPV integration can precede overall aneuploidization in premalignant lesions of the uterine cervix. The presence of TERC gain in eight of ten diploid lesions with integrated HPV is of particular note and supports previous data suggesting that squamous neoplasia of the cervix develops via two different pathways, one of which is associated with overall numerical chromosome abnormalities [6].

It is generally accepted that nearly all CIN 2/3 lesions are HPV-positive when examined by sensitive PCR protocols. Because of its general distribution in these lesions, the presence of oncogenic HPV cannot be applied diagnostically to predict progression. Integration of oncogenic HPV into the host genome is, however, an important step in the progression of CIN 2/3 to cervical carcinoma. Our earlier studies showed a strong correlation between the physical status of HPV and progression to (micro)invasive carcinoma [8,32]. This integration is now shown to be associated with the presence of chromosome 3 and 7 aberrations. In solitary CIN 2/3, DNA diploidy and/or tetraploidy were predominantly seen, while aneuploidy was frequent in CIN 3&mCA and invCA [18,20]. This is in line with previous studies, with the exception that tetrasomy was previously reported to be characteristic for CIN 1 lesions [18]. Our data show that episomal HPV is generally associated with overall DNA diploidy/tetraploidy and that integration of HPV is associated with chromosomal aneusomies. This supports the idea that integration of the virus leads to or accompanies genomic instability. Graham et al reported that CIN 2/3 and invCA are frequently DNA diploid as determined by FISH, utilizing several centromere probes [6]. It was suggested that two different pathways for the development of these lesions exist: one with initial tetrasomy with subsequent loss of chromosomes, and a second that does not involve persistent tetrasomy. Integration of the virus in the latter group may explain our observed diploid CIN 3&mCA cases. In about 15% of our lesions, the chromosome copy number exceeded 5, which is relatively high in comparison to other studies targeting individual chromosomes [6,12,18]. Hypertetrasomy was, however, frequently recognized by others in CIN III and invasive carcinomas [7,38,39]. In these studies, however, the percentage of cells with these high copy numbers was low. Because our FISH evaluation is based on the determination of the maximum signal copy number, screening of tissue areas with polyploid cells may result in an overestimation of the chromosomal copy number.

Parallel to changes in DNA ploidy, copy number aberrations for TERC as well as relative gain of TERC correlated strongly with progression of CIN 2/3 to (micro)invasive carcinoma. Gain of the TERC gene was detected in 18% of the solitary CIN 2/3 lesions, while in CIN 3&mCA and invCA, gain was seen in 54% and 70% of the cases, respectively. These data support previous studies showing that acquisition of 3q — in our study, extra copies of TERC — correlates with progression of disease [10]. Furthermore, our data demonstrate that this gain is seen in only a small number of solitary CIN 2/3 lesions. TERC gain was seen as a trisomy in about 50% of the cases, which were classified as DNA diploid. With progression, areas within the lesions show polyploidization, a general mechanism for selection of malignant clones, resulting in cells with high chromosome copy numbers or cells with chromosomal imbalances. In this respect, it is noteworthy that several CIN 3&mCA lesions exhibited a single HPV spot (single integration site) and trisomy for TERC, while in two of the invCA, multiple HPV spots and high copies of TERC were seen. We can hypothesize that in this case, the stable ratio between TERC and copy number for chromosomes 3 and 7 is the result of polyploidization.

It is tempting to speculate that integration of the virus precedes TERC gain in the development of an...
invasive carcinoma from a premalignant lesion. The literature provides conflicting data. As stated above, it is accepted that viral integration occurs somewhere during progression from a premalignant lesion to an invCA [1,4]. Some authors claim that nearly all CIN 2/3 lesions harbour integrated virus, while others claim that only a small fraction of these lesions contain integrated virus [15,32]. This controversy is, at least in part, the result of technical problems involved in assessing the physical status of HPV by the different protocols available [15]. The frequency with which we detected integrated virus in solitary CIN 2/3 is in line with the data obtained by Knebel Doeberitz and co-workers, who detected integration of HPV using RT-PCR (the APOT assay). [15,32,40,41] Exclusively episomal or integrated HPVs are perhaps only found at opposite ends of the spectrum, in particular in low-grade premalignant lesions and invCA, respectively, while mixed patterns are found within high-grade premalignant lesions. Our FISH data, focusing on clonal expansion of cells with integrated HPV, showed that these were found in only a minor fraction of high-grade lesions. We suggest that this small fraction of integrated CIN 2/3 lesions could represent the lesions which, if left untreated, will progress to invCA.

Furthermore, we also noticed mixed patterns of HPV with separate areas containing only integrated or episomal HPV and areas where the integrated virus was hidden in a background of episomal HPV copies. In one case, we found TERC gain both in areas with episomes and in areas with integrated HPV, suggesting that TERC gain precedes integration. However, we cannot exclude the possibility that integrated virus is hidden in a background of episomal copies in this case. These questions will only be answered by the analysis of specific groups of cells using objective assays such as APOT and DIPS [4,42]. To date, however, these assays are not routinely applicable to paraffin-embedded tissue material. The observation that integrated virus can be hidden in a background of episomal copies is supported by the recent data from Coleman and co-workers, who showed that episomes can be lost during the selection of cells containing integrated virus [43].

Recently, Ried and co-workers reported that TERC gain in cytological samples from low-grade lesions is an indicator of progression, while absence of gain, including tetrasomy, is an indicator for regression [12]. Unfortunately, these authors provided no data on HPV status. It is difficult to compare this study with our histological data as it is known that the investigation of patients with cyto logically abnormal smears may, on colposcopic and histological evaluation, reveal a normal cervix. These authors suggested that extra copies of TERC render a growth advantage to cervical epithelial cells, culminating in a point of no return in the sequence of malignant transformation. We have speculated that integration of the virus is the point of no return, after which it is very difficult to escape from progression.

The vast majority of CIN 1 lesions regress spontaneously and only very few lesions persist or progress to CIN 2–3. It is estimated that about 10–20% of CIN 2/3 eventually progress to invasive cervical cancer. These frequencies would fit with our observation that HPV integration, a process that runs parallel with gain of TERC (p < 0.005), occurs in only a small fraction of CIN 2/3 lesions.

In conclusion, our data show that gain and aneuploidy of the TERC gene correlate strongly with the transition from CIN 3 to a (micro)invasive carcinoma and are associated with viral integration even in diploid high-grade lesions. Furthermore, both processes can occur in diploid CIN 3 lesions, where trisomy for TERC is typical. With progression to carcinoma, parts of these lesions may undergo polyplloidization, resulting in overall aneuploidy and high TERC gene copy numbers. Genomic integration of HPV and gain of the human telomerase gene TERC appear to be strongly associated with progression. To assess their capacity to identify progressive premalignant cervical lesions or malignant cells in cytological specimens, combined analysis measuring HPV status together with chromosomal ploidy or imbalances, including TERC, should be extensively tested.

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