Clonality and HPV Infection Analysis of Concurrent Glandular and Squamous Lesions and Adenosquamous Carcinomas of the Uterine Cervix

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Key Words: Uterine cervix; Concurrent; Glandular; Squamous; Adenosquamous; Clonality; HPV; Episomal; Integrated

Abstract

We analyzed the clonality and human papillomavirus (HPV) infection status of concurrent glandular and squamous lesions and adenosquamous carcinomas of the uterine cervix to clarify their histogenesis. The glandular and squamous components were clonally different from each other in 7 informative concurrent lesions. HPV was episomal in 2 polyclonal glandular dysplasias (GDs). HPV was in a mixed integrated-episomal form in a monoclonal GD, an adenocarcinoma in situ, and an adenocarcinoma. Both tumor components were monoclonal in origin in 6 adenosquamous carcinomas, with identical patterns of X-chromosomal inactivation and types and physical status of HPV. These results imply that the concurrent glandular and squamous lesions are formed separately, whereas adenosquamous carcinoma is more likely to be a combination tumor of monoclonal origin, and that integration of HPV has an important role in the progression from polyclonal GD through monoclonal expansion to adenocarcinoma in situ and adenocarcinoma.

Cervical intraepithelial neoplasia (CIN) is widely regarded as a precursor lesion to squamous cell carcinoma of the uterine cervix. Infection involving certain high-risk types of the human papillomavirus (HPV) is thought to be a significant coinitiating carcinogenic factor, resulting in their often being detected in cervical adenocarcinoma, adenocarcinoma in situ (AIS), and glandular dysplasia (GD), although the full malignant potential of GD is not yet definitive. CIN lesions are often found adjacent to purely glandular lesions of adenocarcinoma, AIS, and GD. We have adopted the terminology concurrent lesions to describe these adjacent but different cervical tumors. Previous reports have demonstrated that isotypic HPV was detected in both parts of these concurrent glandular and squamous lesions, indicating that, at a minimum, the same type HPV triggered formation of both parts.

Approximately 10% of uterine cervical carcinomas are of the adenosquamous carcinoma type, which are, by definition, composed of a mixture of malignant glandular and squamous epithelial elements. As in the adenomatous lesions, high-risk HPVs have also been detected in 42% to 95% of cervical adenosquamous carcinomas. HPV can persist in infected tissue as a chromosomally integrated form, an extrachromosomal episomal form, or both. When present in cervical carcinoma, the viral genome of the high-risk-type HPV is usually present integrated into the host genome. Viral integration can be demonstrated by 2-dimensional gel-electrophoresis or by quantitatively comparing the copy levels of HPV E2 and E6 gene DNA by real-time polymerase chain reaction (PCR). An earlier study, using 2-dimensional gel-electrophoresis, showed that HPV integration into the tumor cell genome was more...
characteristic of cervical carcinoma than of the precancerous CIN lesions. However, recent studies, using real-time PCR, have suggested that integration of high-risk-type HPV into the host genome actually occurs in a fraction of CIN cases.

We have previously demonstrated that HPV integration triggers monoclonal expansion of dysplastic cells in CIN and that such CIN lesions were more likely to persist or progress. The physical status of HPV DNA in related AIS and GD, however, has yet to be similarly explored. In addition, the comparison of subtype and physical status of HPV in the glandular and squamous components of adenosquamous carcinoma and concurrent glandular and squamous lesions has not been fully investigated. A previous study has shown that, in 2 cases of adenosquamous carcinomas, losses of heterozygosity of microsatellite markers for 9 separate chromosomes were similar in each component, indicating that the entire adenosquamous carcinoma was most likely monoclonal. Because the clonality of both components of an adenosquamous carcinoma, and of the more commonly seen concurrent glandular and squamous presumptive precursor lesions, has yet to be similarly analyzed, it remains to be proven whether concurrent glandular and squamous lesions are actually the precursor of adenosquamous carcinoma.

A fundamental principle held by most tumor biologists is the belief that most neoplastic tissues are composed of clonal cell populations. Although many methods have been used to investigate the clonal composition of normal tissues and tumors, the most consistently informative cell-origin marker is the pattern of X-chromosomal inactivation in XX females. During early embryogenesis in the female, the paternally derived or the maternally derived X chromosome in each cell is randomly inactivated by methylation of the majority of its cytosine residues of CpGs, and the choice, once made, is stable through subsequent cycles of cell division. Thus, normal tissues of females show a random pattern of X-chromosomal inactivation. By contrast, neoplastic tissues from female subjects display a uniform pattern of X-chromosomal inactivation, consistent with the monoclonal theory of composition. By using this theory, Wada et al demonstrated that roughly 90% of carcinosarcomas of the uterine corpus were combination phenotype tumors derived from a single cell of origin and that the rest were “collision tumors” which “coincidentally occurred, independently and concurrently.”

In the present study, we evaluated the X-chromosomal clonality and the physical status of HPV infection (episomal, integrated, or mixed form) of the glandular and squamous components of concurrent glandular and squamous lesions and of adenosquamous carcinoma. We hope to provide a clarification of the histogenesis of these 2 lesions as to whether the former lesions are a precursor to the latter.

Materials and Methods

During the 5-year period 1999 to 2004, 8 surgical cases of uterine cervical adenosquamous carcinoma and 19 cases of glandular lesions with concurrent CINs of the cervix were histologically diagnosed and collected at the Osaka University Hospital, Osaka, Japan. From these cases, 7 cases of adenosquamous carcinoma and 8 cases of glandular lesions with concurrent CINs were included in the study. Following obtainment of informed consent, these tissue samples were used for further molecular analysis.

Laser-Capture Microdissection and DNA Extraction

Formalin-fixed, paraffin-embedded 4-µm sections were cut at 5 serial levels, and the sections were placed on glass slides and stained with H&E. After staining, the sections were dehydrated in graded alcohols, deparaffinized in xylene, and air dried. Five sections of each sample were microdissected using an LM200 Laser-Capture Microdissection system (Arcturus Engineering, Santa Clara, CA). Each glandular and squamous part was independently microdissected. DNA was extracted from each microdissected sample by Proteinase K digestion followed by phenol-chloroform extraction, as previously described. Corresponding adjacent normal epithelial or stromal cells were extracted from the same slide and used as internal control cells for the clonality analysis. For the study, 8 cases of concurrent glandular-and-squamous lesions and 7 cases of adenosquamous carcinoma were analyzed.

Analysis of X-Chromosomal Inactivation by PCR Amplification

The X-linked human androgen receptor gene, HUMARA, which maps to X-cen-q13, contains in its first exon an intronless trinucleotide-repeat encoding from 11-31 glycine residues. HUMARA is allelic-polymorphic in 90% of women. The methylation status of HhaI restriction sites, located 100 base pairs upstream of the trinucleotide-repeat element, correlates with the X-chromosomal inactivation state of the surrounding X chromosome. The patterns of X-chromosomal inactivation within the adjacent lesions were analyzed and compared by methylation-sensitive restriction endonuclease HhaI treatment of the extracted DNA, followed by PCR amplification of the HUMARA target, as previously reported. Briefly, DNA extracted from paraffin-embedded archival sections was digested with 2 U of HhaI in 10 µL of digestion buffer at 37°C for 12 hours. After terminating digestion by incubation at 95°C for 10 minutes, the DNAs were reextracted with phenol-chloroform, precipitated with ethanol, and used as PCR templates. HhaI-undigested DNA was retained as the control sample.

Nested PCR was performed to increase the sensitivity of PCR amplification, using primers and conditions for PCR.
amplification, as previously described.26 One microliter of the outer-primer set of amplified product was subjected to a second round of nested PCR amplification under the conditions described above. The PCR products were processed on an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer, Foster City, CA), and the results were analyzed using ABI PRISM GeneScan Analysis Software (version 2.1). PCR amplification was also performed on DNA extracted from matching histologically normal endometrial epithelial cells or stromal tissue from each case. All samples were run in duplicate to avoid misinterpretation and laboratory error and to demonstrate reproducibility.

**Detection and Typing of HPV by PCR–Restriction Fragment Length Polymorphism**

The presence of HPV was examined by PCR to generate amplified DNA fragments within the open reading frames (ORFs) of E6 and E7 using the following consensus-sequence primers: pU-1M, 5’-TGCTAAAAACCGTTGTTCC-3’; pU-31B, 5’-TGCTAAATCGGTGTCACCTG-3’; and pU-2R, 5’-GAGCTGTCGCTTAATTGCTC-3’, as described.27 Low-risk HPVs (types 6 and 11) were detected by amplification with primers pU-2R and pU-31B. High-risk HPVs (types 16, 18, 31, 33, 35, 52b, and 58) were amplified using primers pU-2R and pU-1M.

Five microliters of the PCR products were independently digested with 4 U of the restriction enzymes Rsal, AvaII, or Sau3AI, for 3 hours, according to procedures described by the manufacturer (TaKaRa, Tokyo, Japan). The digestion products were fractionated on 4% nondenaturing agarose gels, stained with ethidium bromide, and photographed.

**Analysis of HPV-16 and HPV-18 Physical Status by Real-Time PCR**

The physical status (integrated vs nonintegrated form) of HPV-16 and HPV-18 was examined by real-time PCR amplification of HPV E2 and E6 ORFs. Primers for the E2 ORF were designed to amplify the region that is usually deleted in adenocarcinoma with CIN had both histiotypes homozygous for the HUMARA allele. Blocked amplification of 1 of the 2 alleles resulted in the detection of 2 major clonality analysis.

For the HPV-18 E2 ORF, the primers were as follows: sense, 5’-ATATCATCCACCTGCAATTGG-3’ (nucleotide position, 3762-3782); and antisense, 5’-TTCTTTTGTTTTCCATATGTTGAATTTACA-3’ (nucleotide position, 3818-3846). For the E6 ORF, the sense primer was 5’-GAACCTACAGAGGTATTTGAATTTGC-3’ (nucleotide position 221-247) and the antisense primer, 5’-TGTTCTCATAAGCTGTAATTTAGT-3’ (nucleotide position, 342-369). The TaqMan probes were as follows: 5’-FAM-ATTCCTGTGTTTCTATTGCACTG-TGATAGCATAAATGTT-3’ for the E2 ORF and 5’-FAM-CCGATGCTGATGCCATCTAATGTT-3’ for the E6 ORF.

The 50 µL of PCR reaction mixture consisted of 5 µL of template DNA, 5 µmol/L of each sense and antisense primer, 2 µmol/L of TaqMan probe, and 25 µL of the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). The amplification conditions were as follows: 2 minutes at 50°C, 10 minutes at 95°C, followed by 50 cycles of a 2-step cycle of 95°C for 15 seconds and 60°C for 60 seconds. The cutoff value for the ratio of E2 to E6 copy numbers was set at 0.5 on the basis of the reliability of the real-time PCR. The E2 and E6 ORFs of HPV-16 were quantified with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Cloned HPV-16 DNA and cloned HPV-18 DNA, which contained the whole genome of HPV-16 and HPV-18, respectively, and PZE67 and pU18E67, which contained the E6-E7 ORFs of HPV-16 and HPV-18, respectively,31,32 were provided by M. Yutsudo, MD, PhD (Department of Tumor Virology, Research Institute for Microbial Disease, Osaka University), and used as control samples for detecting the episomal and integrated forms of HPV.

**Results**

**Clonal Analysis of Concurrent Glandular and Squamous Lesions**

PCR amplification was performed to generate products surrounding the highly polymorphic CAG repeat within exon 1 of the HUMARA gene, using DNA extracted from microdissected tissues as templates. PCR amplification of undigested DNA resulted in the detection of 2 major HUMARA amplification products of almost identical length in 13 of the 15 cases included in this portion of our study. One case of concurrent adenocarcinoma with CIN had both histotypes homozygous for the HUMARA target and, thus, had to be excluded from clonality analysis.

**Pretreatment of genomic DNA with the methylation-sensitive restriction endonuclease HhaI blocked PCR amplification of any unmethylated (transcription-active) HUMARA allele. Blocked amplification of 1 of the 2 HUMARA alleles...**
by HhaI pretreatment of the genomic DNA was interpreted as evidence of a uniform pattern of X-chromosomal inactivation consistent with a monoclonal tumor cell population. By contrast, if HhaI pretreatment of the genomic DNA failed to block amplification of either band [Figure 1], the case was considered polyclonal.\textsuperscript{20,23-25} Among 7 informative cases of concurrent glandular and squamous lesions, all 3 cases of adenocarcinoma with AIS (cases 1, 3, and 4) and 1 case (case 7) of 4 GDs were monoclonal in composition, and 5 of 7 concurrent CINs (cases 1 and 3-6) were also monoclonal [Table 1].

**Figure 1** Clonality analysis of cervical intraepithelial neoplasia by a polymerase chain reaction (PCR)-based method. GeneScan images of amplification products of representative cases are shown. A, Noninformative case. PCR amplification of undigested (top) or digested (bottom) genomic DNA resulted in amplification of a single HUMARA peak in both, indicating that this case was homozygous in the HUMARA gene locus and was, therefore, noninformative for clonality analysis. B and C, Monoclonal tumors A and B are informative cases showing evidence of monoclonal composition. PCR of their undigested DNA resulted in amplification of 2 peaks. Pretreatment of genomic DNA, with HhaI digestion followed by PCR amplification, in each case blocked amplification of the larger (monoclonal A) or smaller (monoclonal B) of the 2 peaks, indicating a uniform pattern of X-chromosomal inactivation. D, Informative cases showing evidence of polyclonal composition. PCR of undigested or digested DNA resulted in amplification of 2 peaks.

**HPV Infection Analysis of Concurrent Glandular and Squamous Lesions**

Testing for HPV DNA was performed by PCR–restriction fragment length polymorphism analysis. High-risk-type HPVs were detected in 7 (88%) of 8 cases. Of these 7 cases, HPV-16 was detected in 3 (43%) (cases 3, 5, and 6), HPV-18 was present in 4 (57%) (cases 1, 2, 6, and 7), and HPV-52 was found in a single case (14%) (case 4). Coinfection by HPV-16 and HPV-18 was detected in 1 case (14%) of concurrent GD and CIN (case 6). Low-risk types of HPV were not detected in any lesions.

The physical status of the HPV-16 and HPV-18 genome was examined by real-time PCR amplification of the HPV E2 and E6 ORFs. Primers for E2 ORF were designed to amplify the ORF region, which is usually deleted on the viral integration into the host genome in cervical carcinoma.\textsuperscript{28,29} The detection of both E2 and E6 at equivalent levels indicated that the HPV genome remained in its episomal form [Figure 2A]. The detection of E6 but not E2 was interpreted as evidence of complete HPV integration into the host genome, with no residual episomal form [Figure 2B]. By contrast, the detection of E6 at relatively high levels with a low level of amplification of E2 was interpreted as an indication that the HPV viral genome was present as a mixture of integrated and episomal forms [Figure 2C].\textsuperscript{17,33}

When the control plasmids pZ-E67 and pU18E6\textsuperscript{7,31,32} were used as the PCR template for HPV-16 and HPV-18, respectively, amplification of E6 but not E2 was observed. These plasmids served as internal control samples for the integrated types of HPV-16 and HPV-18, respectively. HPV DNA was detected in glandular parts of concurrent lesions as an integrated type in 1 case (case 1), as a mixed type in 3 cases (cases 2, 3, and 7), and as an episomal type in 2 cases (cases 5 and 6), whereas HPV DNA was detected in the squamous parts of concurrent lesions as a mixed type in 5 cases (cases 2, 3, and 5-7), and as an episomal type in 1 case (case 6).

**Comparison of Glandular and Squamous Parts of Concurrent Glandular and Squamous Lesions**

Among 8 cases of concurrent glandular and squamous lesions, 7 cases were informative for clonal analysis. These cases included 1 case of concurrent adenocarcinoma and CIN (case 1), 2 cases of concurrent AIS and CIN (cases 3 and 4), and 4 cases of concurrent GD and CIN (cases 5-8). In the 3 cases of concurrent adenocarcinoma or AIS and CIN (cases 1, 3, and 4), the glandular and squamous parts were monoclonal within each component, but their patterns of X-chromosomal inactivation were different, indicating that the 2 adjoining lesions had separate origins and that these lesions then developed separately. Four cases of concurrent GD and CIN were analyzed. The clonal status of one
part was demonstrated to be polyclonal and that of the other to be monoclonal in 3 (cases 5-7). In the remaining case (case 8), both parts were polyclonal. The evidence suggests separate cells of origin for both parts of these concurrent tumors.

HPV was not detectable in case 8. In the other 7 cases, high-risk-type HPVs were detected (cases 1-7), and in both parts the type of HPV was shown to be identical. However, in 3 cases (cases 1, 5, and 6) the physical status of the HPV in the 2 parts was different. An example (case 3) is shown in Figure 3 and Image 1.

### Table 1

<table>
<thead>
<tr>
<th>Lesion/Case No./Histologic Diagnosis</th>
<th>Clonality</th>
<th>HPV Type (Physical Status)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concurrent glandular and squamous lesions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Adenocarcinoma (endocervical type)</td>
<td>Monoclonal A</td>
<td>18 (integrated)</td>
</tr>
<tr>
<td>CIN 3</td>
<td>Monoclonal B</td>
<td>18 (integrated)</td>
</tr>
<tr>
<td>2 Adenocarcinoma (endocervical type)</td>
<td>NI</td>
<td>18 (mixed)</td>
</tr>
<tr>
<td>CIN 2</td>
<td>18 (mixed)</td>
<td></td>
</tr>
<tr>
<td>3 AIS (endocervical type)</td>
<td>Monoclonal A</td>
<td>16 (mixed)</td>
</tr>
<tr>
<td>CIN 3</td>
<td>Monoclonal B</td>
<td>16 (mixed)</td>
</tr>
<tr>
<td>4 AIS (endocervical type)</td>
<td>Monoclonal B</td>
<td>52</td>
</tr>
<tr>
<td>CIN 3</td>
<td>Monoclonal A</td>
<td>52</td>
</tr>
<tr>
<td>5 GD</td>
<td>Polyclonal</td>
<td>16 (episomal)</td>
</tr>
<tr>
<td>CIN 3</td>
<td>Monoclonal B</td>
<td>16 (mixed)</td>
</tr>
<tr>
<td>6 GD</td>
<td>Polyclonal</td>
<td>16 (episomal); 18 (episomal)</td>
</tr>
<tr>
<td>CIN 2</td>
<td>Monoclonal A</td>
<td>16 (mixed); 18 (mixed)</td>
</tr>
<tr>
<td>7 GD</td>
<td>Monoclonal B</td>
<td>18 (mixed)</td>
</tr>
<tr>
<td>CIN 2</td>
<td>Polyclonal</td>
<td>18 (mixed)</td>
</tr>
<tr>
<td>8 GD</td>
<td>Polyclonal</td>
<td>–</td>
</tr>
<tr>
<td>CIN 2</td>
<td>Polyclonal</td>
<td>–</td>
</tr>
<tr>
<td>Adenosquamous carcinoma</td>
<td></td>
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</tr>
<tr>
<td>9 Adenocarcinoma</td>
<td>Monoclonal A</td>
<td>18 (mixed)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>Monoclonal A</td>
<td>18 (mixed)</td>
</tr>
<tr>
<td>10 Adenocarcinoma</td>
<td>Monoclonal A</td>
<td>18 (integrated)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>Monoclonal A</td>
<td>18 (integrated)</td>
</tr>
<tr>
<td>11 Adenocarcinoma</td>
<td>Monoclonal B</td>
<td>16 (episomal)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>Monoclonal B</td>
<td>16 (episomal)</td>
</tr>
<tr>
<td>12 Adenocarcinoma</td>
<td>Monoclonal B</td>
<td>16 (mixed)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>Monoclonal B</td>
<td>16 (mixed)</td>
</tr>
<tr>
<td>13 Adenocarcinoma</td>
<td>Monoclonal B</td>
<td>18 (mixed)</td>
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<tr>
<td>Squamous cell carcinoma</td>
<td>Monoclonal B</td>
<td>18 (mixed)</td>
</tr>
<tr>
<td>14 Adenocarcinoma</td>
<td>Monoclonal B</td>
<td>–</td>
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<tr>
<td>Squamous cell carcinoma</td>
<td>Monoclonal B</td>
<td>–</td>
</tr>
<tr>
<td>15 Adenocarcinoma</td>
<td>NI</td>
<td>16 (integrated)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>16 (integrated)</td>
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</table>

*Clonal status and HPV infection were analyzed, specifically for HPV-16 and HPV-18. The physical status of the HPV DNA is also shown. Mixed, episomal, and integrated forms of HPV; monoclonal A, small allele inactivated; monoclonal B, larger allele inactivated; NI, noninformative for clonality analysis; –, HPV not detected.

**Clonal and HPV Infection Analysis of Adenosquamous Carcinomas**

One case of adenosquamous carcinoma was not informative for clonality analysis. In all 6 informative adenosquamous carcinomas (cases 9-14), the glandular and squamous components were demonstrated to be monoclonal. High-risk-type HPVs were detected in 6 (86%) of 7 cases. Low-risk-types of HPV were not detected in any lesions. HPV-16 was detected in 3 (50%; cases 11, 12, and 15), and HPV-18 was present in 3 cases (50%; cases 9, 10, and 13). In the glandular and
**Figure 2** Real-time polymerase chain reaction (PCR) analysis of the physical status of human papillomaviruses. Run profiles of ΔRn (normalized fluorescence emission with subtracted baseline fluorescence) vs PCR cycles in 3 types are shown: **A**, Episomal form, equivalent copy numbers of E2 and E6 were detected. **B**, Integrated form, only E6 amplification was detected, without E2 amplification. **C**, Mixed form: copy numbers of E2 were fewer than of E6 but were not undetectable.

**Figure 3** Analysis of clonality (**A**) and human papillomavirus (HPV) infection (**B**). Clonality of adenocarcinoma in situ (AIS) and cervical intraepithelial neoplasia (CIN) 3 was different in the 2 lesions (monoclonal A and monoclonal B, respectively). However, HPV-16 was detected as a mixed type in AIS (left) and CIN 3 (right).
squamous portions of adenosquamous carcinomas, the physical status of HPV DNA was demonstrated to be identical; HPV was of the integrated type in 2 cases (cases 10 and 15), mixed type in 3 cases (cases 9, 12, and 13), and episomal type in 1 case (case 11).

Comparison of Glandular and Squamous Components of Adenosquamous Carcinomas

Among 7 cases of adenosquamous carcinoma, 6 cases were informative for clonal analysis (cases 9-14). The clonal status of the adenocarcinoma and squamous cell carcinoma components of each was monoclonal and identical for patterns of X-chromosomal inactivation, implying that each adenosquamous carcinoma was a combination phenotype tumor, deriving from a single cell (Table 1). In HPV+ cases (cases 9-13 and 15), the type and physical status of HPV were identical in both components, implying a homogeneous composition of the adenosquamous carcinoma. One example (case 13) is shown in Figure 4 and Image 2.

Figure 4 Analysis of clonality (A) and human papillomavirus (HPV) infection (B). Clonality of both components was monoclonal. HPV-18 was detected as a mixed type in adenosquamous (left) and squamous cell (right) carcinoma.

Image 1 (Case 3) Concurrent adenocarcinoma in situ and cervical intraepithelial neoplasia 3 (H&E, ×40).

Image 2 (Case 3) Concurrent adenocarcinoma in situ and cervical intraepithelial neoplasia 3 (H&E, ×40).
Integration of high-risk-type HPVs, especially HPV-18 and HPV-16, is thought to be a potent trigger of carcinogenesis in cervical adenocarcinoma. In HPV− adenocarcinomas, loss of expression due to hypermethylation of the promoter regions of 14-3-3-σ, one of the G2/M checkpoint genes, and of RASSF1A, a putative tumor suppressor gene, was more frequently observed.

Adenosquamous carcinoma is a form of carcinoma exhibiting glandular and squamous differentiation. Adenocarcinomas coexisting with squamous cell carcinoma or intraepithelial neoplasia as collision tumors are excluded from the diagnosis of adenosquamous carcinoma.

Clonality analysis of adenosquamous carcinoma of the lung, using a HUMARA method similar to the method used in this study, demonstrated an identical pattern of X-chromosomal inactivation in both components of adenocarcinoma and squamous cell carcinoma in 2 separate cases, supporting their monoclonal origins. Another report, based on genetic alterations of p53, K-ras, and chromosomal abnormalities at 9p21 and 9q31-32 and immunohistochemical reactions for p53, squamous cell carcinoma–related antigen, and mucin 1 proteins, gave evidence that both tumor components were identical in origin in 11 of 12 cases of adenosquamous carcinoma of the lung.

For esophageal adenosquamous carcinoma, van Rees et al demonstrated a loss of the same alleles at 4 microsatellite markers on 9p and that a missense mutation within p53 was identical in both components, but that 1 shift at a microsatellite marker on 16q was different in the 2 components in a single case. They thus concluded that adenosquamous carcinoma of esophagus had a monoclonal origin and that divergence occurred late in the tumorigenesis process.

Until now, there has been no demonstration as to whether adenosquamous carcinoma of the uterine cervix is a combination tumor derived from a single cell. Moreover, the character of the precursor lesion of adenosquamous carcinoma is still unclear, whereas CIN is widely regarded as the precursor lesion of squamous cell carcinoma. We have previously demonstrated that integration of high-risk-type HPVs is a trigger of monoclonal expansion and progression of CIN. AIS is defined as a lesion in which normally situated glands are partially or wholly replaced by cytologically malignant epithelial cells and is regarded as a precursor of adenocarcinoma. A similar type of HPV was more frequently observed.

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detected in glandular and squamous parts in concurrent glandular and squamous lesions.\textsuperscript{7-9,47} The clonal status and HPV physical type of both tumor parts has not been analyzed, and the histogenesis of concurrent glandular and squamous lesions remains unclear.

In this study, 2 cases of concurrent adenocarcinoma and CIN, 2 cases of concurrent AIS and CIN, 4 cases of concurrent GD and CIN, and 7 cases of adenosquamous carcinoma of the uterine cervix were evaluated. Representative H&E-stained sections of these lesions were shown in Image 1, Image 2, and Image 3. We first investigated the malignant potential of GD and AIS. GD has been proposed as a diagnostic term based on the assumption that glandular lesions progress through a series of lesions of distinctive morphologic features as they acquire the genetic and phenotypic changes of carcinoma.\textsuperscript{48} Although GD closely resembles AIS, it differs in that the nuclei are not cytologically malignant and mitotic figures are less numerous. Nuclear hyperchromatism and enlargement of the cells is prominent.\textsuperscript{4,48}

In our study, it was demonstrated that integration of high-risk-type HPVs occurred in some fractions of AIS and GD. Clonality analysis revealed the 2 cases of AIS and 1 of 4 cases of GD were monoclonal in composition, indicating that AIS and a small fraction of GDs have a neoplastic character and that most GDs are nonneoplastic, reactive lesions. In each of the informative glandular cases provided for analysis of clonality and HPV physical status, HPV existed as an episomal type in 2 polyclonal GDs and a mixed or integrated type in 3 monoclonal lesions, including 1 adenocarcinoma, 1 AIS, and 1 GD. These results may suggest that integration of high-risk-type HPVs has an important role in progression from reactive and polyclonal GD through monoclonal expansion to AIS and adenocarcinoma, supporting the theoretical concept of GD.

We further investigated the relationship between the 2 parts of concurrent glandular and squamous lesions and their relationship to adenosquamous carcinoma. In 7 informative cases, the clonal status of the glandular and squamous lesions were demonstrated to be monoclonal, but the pattern of X-chromosomal inactivation was different in 3 cases, including a single case of concurrent adenocarcinoma and CIN and 2 cases of concurrent AIS and CIN. Either or both lesions were polyclonal in the other 4 cases of concurrent GD and CIN, indicating that the concurrent glandular and squamous lesions were formed separately and not derived from a single common cell. The types of HPV detected in the glandular and squamous parts of all concurrent lesions were identical in all 8 HPV+ cases. However, the physical status of HPV-16 and HPV-18 in glandular parts was different from that of the squamous parts in 3 cases. These HPV infection results are compatible with a separate histogenesis of concurrent glandular and squamous lesions. Taken together, we can conclude that the concurrent glandular and squamous lesions develop separately from the same type HPVs.

On the other hand, in 6 informative cases of adenosquamous carcinoma, the clonal status of each component of adenocarcinoma and squamous cell carcinoma was monoclonal in composition and the patterns of X-chromosomal inactivation were identical, as were the type and physical status of HPV in both components of the 6 HPV+ cases. X-chromosomal inactivation is caused by random methylation of the paternal or maternal allele. The possibility of inactivation of the paternally or maternally derived X chromosome is 50:50 in a cell and, subsequently, in any neoplasia that consists of monoclonal expansive cells. The coincidence of an X-inactivation pattern that is paternal or maternal within 2 different neoplasias in a person is 50:50 in the same way. If an adenosquamous carcinoma is a collision tumor of an adenocarcinoma and a squamous cell carcinoma, the odds of a coincidence of the patterns of X-chromosomal inactivation in all 6 informative cases of adenosquamous carcinoma can be calculated to be 1/2.\textsuperscript{6} Moreover, the same subtype and physical status of HPV were detected in the adenocarcinoma and squamous cell carcinoma portions in all 6 HPV+ cases. Taken together, it may be possible to deduce that adenosquamous carcinoma is a combination tumor caused by monoclonal cell expansion of a single original cell. Although a difference in the X-chromosomal inactivation pattern can disprove a shared clonality, the presence of a shared pattern does not prove the opposite, as there is the 50:50 random possibility they will be the same. Therefore, we cannot deny the possibility of coincidence of the clonal expansion pattern and HPV infection status, so further study is necessary to clarify the hypothesis.

Recently, cancer stem cells have been implicated in the development, recurrence, and chemotherapy/radiotherapy resistance of various kinds of solid tumors.\textsuperscript{49-51} Cancer stem cells are believed to have the inherent ability to self-renew and proliferate, allowing transforming mutations to be perpetuated. Our findings imply that adenosquamous carcinoma is derived from a single stem cell, which has a pluripotency to differentiate into both squamous and glandular cells.

In this study, we showed that many concurrent glandular and squamous lesions are collision tumors formed separately by the same type of HPV and that adenosquamous carcinoma is possibly a combination tumor. These results may suggest that the glandular and squamous parts of concurrent lesions do not develop into the glandular and squamous components of adenosquamous carcinoma, respectively; however we cannot deny the possibility that adenosquamous carcinoma is derived from the glandular or squamous part of the concurrent glandular and squamous lesions. A previous study proposed that stratified mucin-producing intraepithelial
Image 3: A (Case 1), Adenocarcinoma (endocervical type). B (Case 5), Glandular dysplasia (GD). C (Case 6), GD. D (Case 7), GD. E (Case 8), GD (A-E, H&E, ×100).
lesions are potential precursors for adenosquamous carcinomas. Further investigation is necessary to clarify the entire mechanism of development of adenosquamous carcinoma of the uterine cervix.

References


