Type-Dependent Integration Frequency of Human Papillomavirus Genomes in Cervical Lesions

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Abstract

Chromosomal integration of high-risk human papillomavirus (HR-HPV) genomes is believed to represent a significant event in the pathogenesis of cervical cancer associated with progression from preneoplastic lesions to invasive carcinomas. This hypothesis is based on experimental data suggesting that integration-dependent disruption of HR-HPV E2 gene functions is important to achieve neoplastic transformation and on clinical data gathered by analyzing lesions induced by human papillomavirus (HPV) 16 and 18 that revealed integrated viral genome copies in the vast majority of cervical cancer cells. However, a substantial fraction of cervical cancers is associated with other HR-HPV types for which virtually no data concerning their integration status have been reported so far. Here, we compared integration frequencies of the five most common oncogenic HPV types (HPV16, 18, 31, 33, and 45) in a series of 835 cervical samples using a specific mRNA-based PCR assay (Amplification of Papillomavirus Oncogene Transcripts). Most precancerous lesions displayed exclusively episomal viral genomes, whereas 62% of the carcinomas had integrated viral genomes. However, the frequency of integrated HR-HPV genomes showed marked differences for individual HR-HPV types. HPV16, 18, and 45 were found substantially more often in the integrated state compared with HPV types 31 and 33. The analysis of the median age of patients with high-grade precancerous lesions and invasive cancers suggests that precancers induced by HPV types 18, 16, and 45 progress to invasive cervical cancer in substantially less time compared with precancers induced by HPV types 31 and 33. These findings suggest that integration of oncogenic HPV genomes in cervical lesions is a consequence rather than the cause of chromosomal instability induced by deregulated HR-HPV *E6-E7* oncogene expression. Distinct HR-HPV types apparently provoke chromosomal instability in their host cells to a different extent than is reflected by their integration frequencies in advanced lesions and the time required for CIN 3 lesions to progress to invasive cancer. [Cancer Res 2008;68(1):307–13]

Introduction

Infections of the female anogenital tract by oncogenic high-risk type human papillomaviruses (HR-HPV) may result in premalignant and malignant lesions particularly at the transformation zone of the uterine cervix. It is now widely accepted that almost all cervical carcinomas and the majority of vaginal, vulval, penile, and anal cancers arise as consequence of persistent infections with about 13 different HR-HPVs (1). Most of these human papillomavirus (HPV) types show phylogenetic similarities to either HPV16 (31, 33, 35, 52, and 58) or HPV 18 (39, 45, 59, and 68; ref. 2). Although the prevalence of specific HR-HPV types in invasive cancers varies in different geographic regions, HPV16 and HPV18 are clearly the predominant types in cancerous lesions accounting for about 70% of all cases worldwide (1).

HR-HPVs infect basal cells of the epithelium. Initially, the viral genome is replicated together with the host genome without triggering high level viral genome replication and capsid production in infected basal cells. These latent infections may switch to *active* virus replication in terminally differentiated cells of the intermediate and superficial epithelial layers (3, 4).

About 90% of HR-HPV infections are self-limited and regress spontaneously within several months (5, 6). In about 10% of the cases, however, the infection persists and may progress to a transforming HR-HPV infection that induces outgrowth of high-grade preneoplastic lesions or invasive cancers. More recent data indicate that the risk of conversion from an acute infection to the transforming infection is substantially different for individual HR-HPV types (7–9). On the molecular level, the conversion of an *acute HPV-infection* into a *transforming HPV-infection* is characterized by substantially increased expression of the viral oncogenes *E6* and *E7* in the basal cells that retain the capacity to replicate (3, 10). The E6 and E7 proteins induce transformation by interference with host cell factors, such as p53 and retinoblastoma protein that control the cell cycle and apoptosis (11–13), and by disturbing the regulation of the mitotic spindle apparatus. The latter results in chromosome missegregation and breakages inducing severe numerical and structural chromosome alterations and allelic imbalances (14). Taken together, this research clearly documented that deregulated expression of the viral *E6* and *E7* oncogenes induces chromosomal instability and is the predominant hallmark of cervical carcinogenesis. However, the mechanisms that contribute to the deregulated expression of the viral oncogenes are not yet fully understood.

Integration of HPV genomes into host cell chromosomes has been observed in all cervical cancer cell lines, a very high percentage of

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HPV16- and HPV18-positive cervical cancers, and at substantially lower frequencies in high-grade cervical precancerous lesions, however, almost never in early HPV-induced lesions (15–21). This observation suggested that integration of viral genomes is a key event in cervical carcinogenesis and may trigger chromosomal instability.

Three major mechanisms were considered to be important in this respect:

First, disruption of the viral E2 gene in cells with integrated HPV genomes may release inhibition of the viral promoter that controls the oncogenes *E6* and *E7*. This hypothesis was particularly substantiated by the observation that reintroduction of an intact E2 gene in cervical cancer cell lines (HeLa) resulted in growth inhibition due to inhibition of the expression of the E6 and E7 genes (22).

Second, *cis*-acting cellular sequences may influence the viral promoter that controls the transcription of the viral *E6-E7* genes from integrated viral genome copies. Alternatively, cotranscribed cellular sequences may stabilize viral *E6* and *E7* transcripts derived from integrated viral genome copies (23) and, thus, enhance their expression level and oncogenic potential (24, 25).

Third, integrated viral genes may activate cellular oncogenes or inactivate cellular tumor suppressive genes (insertional mutagenesis; refs. 19, 26, 27). Comprehensive data gathered on HPV integration loci in precancers and cancers have shown accumulation of integration sites in transcriptional active and fragile chromosomal regions (19, 20) but does not support the concept that insertional mutagenesis is an essential mechanism in cervical carcinogenesis (16, 18, 28).

Taken together, these aspects suggest that integration of viral genome copies contributes substantially to the neoplastic transformation process. However, the absence of HPV16 DNA integration in some carcinomas implies that integration is not always required for malignant progression. In contrast, the consistent integration of HPV 18 DNA in almost all cervical cancers examined may be related to its greater transforming efficiency *in vitro* and its reported clinical association with more aggressive cervical cancers (21, 29). In most previous studies, HPV integration has been analyzed only for HPV16 and HPV18, whereas data about the integration frequencies of other HR-HPV types in cervical precancerous and cancerous lesions barely exist.

Several recent clinical and epidemiologic studies pointed out that women without cervical abnormalities but detectable infections with HPV types 16 and 18 develop more severe preneoplastic lesions in less time compared with women who are infected with other HR-HPV types, as e.g., HPV 31 or 33 (7, 8). This observation triggered the hypothesis that the relative degree of chromosomal instability that is conferred by distinct HR-HPV types during the progression from HPV infection to invasive cervical cancers may be substantially different. The latter concept suggests that non-HPV16 and 18 HR-HPV types would confer less chromosomal instability than HR-HPV types 16 and 18. This may be reflected by less frequent integration of the respective HR-HPV genomes in advanced cervical lesions and a longer time interval required for progression of preneoplastic lesions to invasive cervical cancers. The aim of the present study was to investigate this hypothesis in a large series of clinical samples that encompass all grades of cervical lesions induced by the five most frequent HR-HPV types. We used the Amplification of Papillomavirus Oncogene Transcripts (APOT) assay (15) that allows to determine the presence or absence of integrated viral genome copies in clinical lesions. Even in the presence of a large excess of episomal nonintegrated viral genomes, single or few integrated viral genome copies can be detected. We compared the integration frequencies of the five most frequent HPV types 16, 18, 31, 33, and 45 and analyzed the median age of the patients at diagnosis of precancer and cancer with respect to HPV type and HPV integration status. The reported data suggest that integration of HR-HPV genomes rather reflects the level of chromosomal instability conferred by distinct HR-HPV types. Although quantitative data on the oncogenic potential of the *E6* and *E7* genes of various HR-HPV types is still lacking, this set of data provides additional molecular epidemiologic proof for the substantially divergent oncogenic power of distinct HR-HPV types in cervical carcinogenesis.

Materials and Methods

Clinical samples. Clinical samples were collected from patients attending the outpatient clinic for cervical dysplasia at the Departments of Obstetrics and Gynecology of the University of Heidelberg (Heidelberg, Germany). Cervical carcinoma samples (The International Federation of Gynecology and Obstetrics; stages IA to IVB) were obtained from patients treated at the Department of Gynecology of the Friedrich-Schiller-Universität, Jena, Germany, at the Norwegian Radium Hospital, and at the Cancer Research Center, Moscow, Russia. In addition, CIN3 samples were obtained from women admitted to Østfold County Hospital, Norway. Biopsy samples were divided into two parts: one was kept for histopathologic analysis and the other one was transferred into cryotubes, immediately shock frozen in liquid nitrogen, and stored until further use. From the patients with normal or low-grade lesions, two independent cervical swab samples were collected using cytobrushes. One of the swab specimens was subjected to routine cytologic analysis (Pap smear) and reviewed by an experienced pathologist. The second brush was transferred into a cryotube (Eppendorf-Netheler-Hinz) and immediately shock frozen in liquid nitrogen.

RNA and DNA isolation. RNA from biopsy and cytobrush samples was isolated using a RNA isolation kit (ToTALLY RNA kit; Ambion) as recommended by the supplier. DNA was extracted using the protocol for simultaneous extraction of RNA and DNA (Ambion Technical Bulletin 161). The DNA was then used for HPV typing of the samples by GP5+/GP6+-EIA according to published protocols (30). For biopsy samples collected in Jena and Moscow, total RNA was isolated using the RNeasy mini kit from Qiagen and the protocol for total RNA extraction from animal tissues. Samples were homogenized by using the QIAshredder. An additional DNase treatment step was included for all samples. For HPV18-, HPV45-, HPV31-, and HPV33-positive samples collected in Norway, nucleic acids were extracted as described earlier (31) and DNA was removed by DNase treatment for 20 min at 70°C (Sigma-Aldrich). Total RNA was eluted in 40 μL of water and stored at −70°C. HPV16-, HPV18-, HPV31-, HPV33-, and HPV45-positive samples were subjected to APOT assay.

Reverse transcription and PCR amplification. Total RNA (0.1–1 μg) was reverse transcribed using an oligo$(dT)_{17}$-primer coupled to a linker sequence p3 (32) and 50 units of Moloney murine leukemia virus reverse transcriptase (SuperScript; Life Technologies) for 1 h at 42°C in a final volume of 20 μL. To control RNA integrity and first-strand cDNA quality, PCR reactions using glyceraldehyde-3-phosphate dehydrogenase–specific primers were performed as described previously (33). First-strand cDNAs encompassing viral oncogene sequences were subsequently amplified by PCR using HPV E7–specific primer [p1] as forward primers (Table 1) and linker p3 as the reverse primer; and 1.5 units of Taq DNA Polymerase (Life Technologies) in a total volume of 50 μL. The reaction mixture was subjected to an initial denaturation step for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at the respective temperatures

Table 1. Primers used for the APOT assay

		HPV E7-specific primers, 5′-3′	Reverse primers, 5′-3′	Ta
1st PCR	P1-HPV31	GCAGAACCGGACACATCC	P3-GACTCGAGTCGACATCG	56
	P1-HPV33	TGAGGATGAAGGCTTGGACC		56
	P1-HPV45	CCCACGAGCCGAACCACAG		56
	P1-HPV16	CGGACAGAGCCCATTACAAT		59
	P1-HPV18	TAGAAAGCTCAGCAGACGACC		61
2nd PCR	P2-HPV31	GTTGTCAGTGTAAGTCTACACTTCG	(dT) P3-GACTCGAGTCGACATGA$(dT)_{17}$	58
	P2-HPV33	CACCACAGTTCGTTTATGTGTCAA		58
	P2-HPV45	GAGAGCTCGGCAGAGGACCTTAG		58
	P2-HPV16	CCTTTTGTTGCAAGTGTGACTCTACG		67
	P2-HPV18	ACGACCTTCGAGCATTCCAGCAG		70

(*Ta*) described in Table 2 for 30 s, elongation at 72°C for 4 min, and a final elongation step at 72°C for 6 min. Five microliters of the amplification product were used as template for nested PCR under identical conditions using forward HPV E7-specific primer [p2] and $(dT)_{17}$-p3 as reverse primer.

Sequence analysis of the viral cellular junctions obtained by APOT. APOT amplification products were visualized by 1.2% agarose gel electrophoresis. Amplimeres that displayed a different size than the major E7-E1^E4 episomal transcript (~1,050 bp in length for HPV16, 31, 33, and 45 and 1,000 bp for HPV18) were suspected to be derived from integrated HPV genomes. PCR products of interest were excised from the gel and extracted using the Qiagen Gel Extraction kit (Qiagen). The corresponding amplimeres were cloned using the TA cloning kit (Invitrogen) or sequenced directly using HPV E7–specific primer [p2]. DNA sequence analysis was performed using the Big-Dye terminator DNA sequencing kit (Perkin-Elmer) and an ABI Prism 310 Genetic Analyser (Applied Biosystems). Sequencing results were analyzed using the BLASTN program provided by the National Cancer Institute, USA.

Statistical analysis. The χ^2 test was used to compare integration frequencies between different HPV types. The Exact Cochran-Armitage Trend test was computed to show a trend of proportions using the Freq procedure in SAS. The two-tailed nonparametric Mann-Whitney test was used to analyze differences in the median age at diagnosis of CIN or cancer, analyses were performed in total and stratified for the underlying HPV type using the SPSS statistical software package. For analysis of multiple comparisons, a Kruskal-Wallis test was computed. For individual comparisons, Dunn's multiple comparison test was performed using the Graphpad statistical software. All *P* values are two tailed; values <0.05 were considered statistically significant.

Results

Prevalence of integrate-derived transcripts in lesions of the uterine cervix. The integration status of the viral genomes was analyzed using the APOT assay, a 3′-RACE PCR assay that allows to differentiate episome from integrate-derived E6-E7 transcripts (15). This assay was initially established to monitor the physical state of HPV16 and 18 genomes and has now been extended to the HR types HPV31, 33, and 45.

In total, 136 samples showed integrate-derived transcripts; in 699 samples, only episomal transcripts were detected. Transcripts suspected to be derived from integrated HPV genome were cloned, sequenced, and compared with the human genome database (National Center for Biotechnology Information). Samples were considered integration positive if fusion transcripts containing viral and cellular sequences could be detected. Database analysis showed that all chromosomes were found to be targeted by HPV integration, confirming the previous reports that there is no preferential HPV integration site in the human genome (18, 34). In 294 samples obtained from subjects with normal histology or cytology results as well as patients with CIN1, APOT analysis revealed exclusively episomal transcripts (Table 2). Five of 172 (3%) CIN 2 lesions and 36 of 216 CIN 3 specimens (17%) displayed integrate-derived transcripts. The proportion of integrate-derived transcripts was substantially higher in invasive cervical carcinoma samples (95 of 153; 62%). The Exact Cochran-Armitage Trend test showed a significant association between the prevalence of integrate-derived transcripts and higher grade of the cervical

Table 2. Integrated HPV oncogene transcripts in cervical samples

	Normal	CIN 1	CIN 2	CIN 3	CxCa	Total	*P*
	n (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n*	
HPV16	0/111 (0)	0/61(0)	5/83 (6)	27/141 (19)	33/60 (55)	456	0.0001
HPV18	0/22 (0)	0/6 (0)	0/13 (0)	0/8 (0)	33/36 (92)	85	0.0001
HPV31	0/22 (0)	0/16 (0)	0/29 (0)	3/29 (10)	2/14 (14)	110	0.0228
HPV33	0/23 (0)	0/20 (0)	0/35 (0)	0/28 (0)	7/19 (37)	125	0.0039
HPV45	0/8 (0)	0/5 (0)	0/12 (0)	6/10 (60)	20/24 (83)	59	0.0001
	0/186 (0)	0/108 (0)	5/172 (3)	36/216 (17)	95/153 (62)	835	0.0001

NOTE: *P*, the exact Cochran-Armitage Trend test.

dysplasia stratified by HPV type and for all samples combined ($P < 0.0001$).

Integration frequency of different HPV types. We observed significant differences when comparing the integration frequencies for single HPV types (Table 2). High integration frequencies were detected in HPV16-, HPV18-, and HPV45-associated cervical carcinoma lesions, whereas lesions induced by HPV31 and HPV33 displayed significantly lower frequencies of integrated viral genome copies. In CIN3 lesions, 60% of HPV45-positive cases showed integrated HPV DNA, followed by HPV16 (19%) and HPV31 (10%), whereas in HPV18- and HPV33-associated CIN 3 lesions, no integrated HPV DNA was detectable (Table 2; χ^2 $P < 0.0001$). In cervical cancer samples, HPV18 exhibited the highest integration frequency with 92% followed by HPV45 (83%), HPV16 (55%), HPV33 (37%), and HPV31 (14%; Table 2; χ^2 $P < 0.0001$).

Integration status of HPV genomes and age of onset of invasive carcinomas. Several experimental studies suggest that integration of HPV DNA might contribute to transformation by allowing for deregulated and enhanced expression of viral oncogenes. Therefore, HPV integration could confer a growth advantage over cells without integrated HPV DNA and thereby accelerate malignant progression to invasive cancer.

To analyze whether the integration status might have an influence on malignant progression, we compared the median age at diagnosis between patients with and without integrated HPV genomes (Fig. 1). In CIN3 cases, there was no significant difference between the median age of patients at diagnosis without (33.9 years) and with integrated viral genome copies (34.0 years). Among the cancer cases, however, patients with integrated HPV DNA had a statistically significant younger median age at diagnosis (43 years) compared with those without integration (52.5 years; $P = 0.017$; Fig. 1), whereas no correlation was found between tumor stage and HPV status (data not shown). Because this effect could be strongly confounded by the different integration frequencies of the underlying HPV types, we performed an analysis stratified by HPV type (Fig. 2). The median age at diagnosis of patients with CIN 3 lesions ranged from 30.8 (HPV33 nonintegrated) to 38 (HPV31 integrated) years and did not show a significant difference with respect to integration status stratified by HPV type (Fig. 2*A*).

The age at diagnosis of patients with invasive carcinomas with integrated or episomal HPV genomes was more heterogeneous with a median age ranging from 41.0 (HPV45 nonintegrated) to 67.6 years (HPV31 nonintegrated; Fig. 2*B*). Although the median age at diagnosis was slightly lower in HPV16-, HPV18-, and HPV33-positive cancer cases with integrated viral genomes compared with cases with episomal genomes, these differences were minor and not statistically significant. In HPV31-positive cases, a high difference of median age at diagnosis between cases without (67.6 years) and with integration (41.3 years) was observed; however, due to the finding of only two integration positive cases, the difference was not significant ($P = 0.088$). In HPV45-positive cases, the patients with integrated HPV DNA had a higher age at diagnosis compared with those without integrated HPV DNA; however, this difference was not statistically significant (Fig. 2*B*).

Based on these data, the overall effect of the integration status on the age of diagnosis seems to be low. However, the median age at diagnosis of invasive cancers stratified by individual HR-HPV types strongly suggests that there is a general effect related to different HR-HPV types. To analyze the influence of the different HPV types on the age at diagnosis, the cases were compared independent of the integration status (Fig. 3). In the CIN3 cases, the only significant age difference was observed between HPV31 and HPV33 (37 and 31 years; $P < 0.01$; Fig. 3*A*). In contrast, the differences in age at diagnosis with respect to HPV types in the cancer cases were more profound. The median age at diagnosis of cervical cancer was 43 to 44 years for HPV16, HPV18, and HPV45, whereas it was up to 20 years higher for the types HPV31 (64 years) and HPV33 (59 years). These age differences were statistically significant ($P < 0.0001$; Fig. 3*B*).

Discussion

Integrated viral genomes have been frequently observed in cervical carcinoma cell lines and primary cancers but only rarely in early precancerous lesions (15, 16, 18, 20, 21, 28). Several reports have suggested that cell clones with integrated viral genome copies in preneoplastic lesions may have a higher risk for progression to invasive carcinomas (15, 21). There is experimental evidence

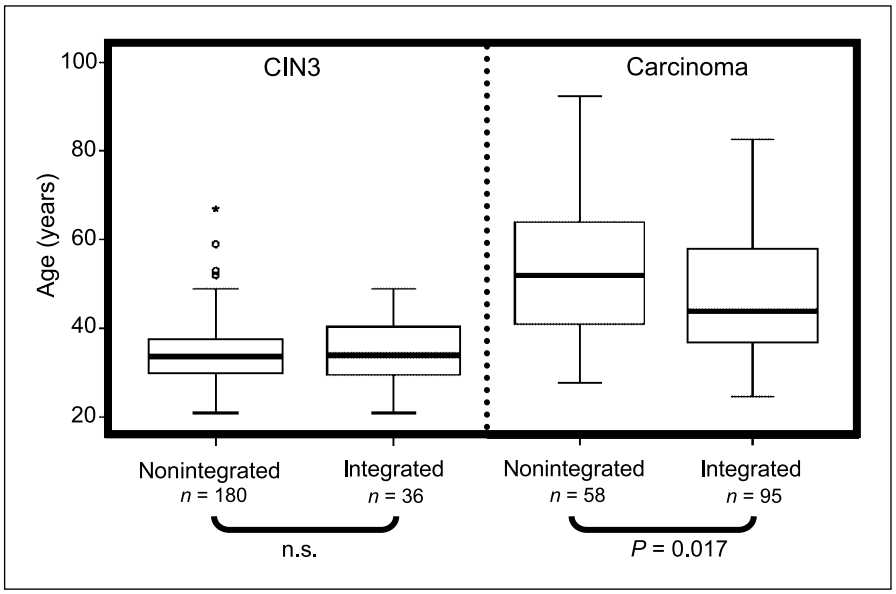

Figure 1. Influence of integration status on age at disease. Box plots of median age at diagnosis of CIN3 and cervical carcinoma by integration status. In carcinomas, a significant difference in median age at diagnosis related to integration status was observed (Mann-Whitney test, $P = 0.017$). *n.s.*, not significant.

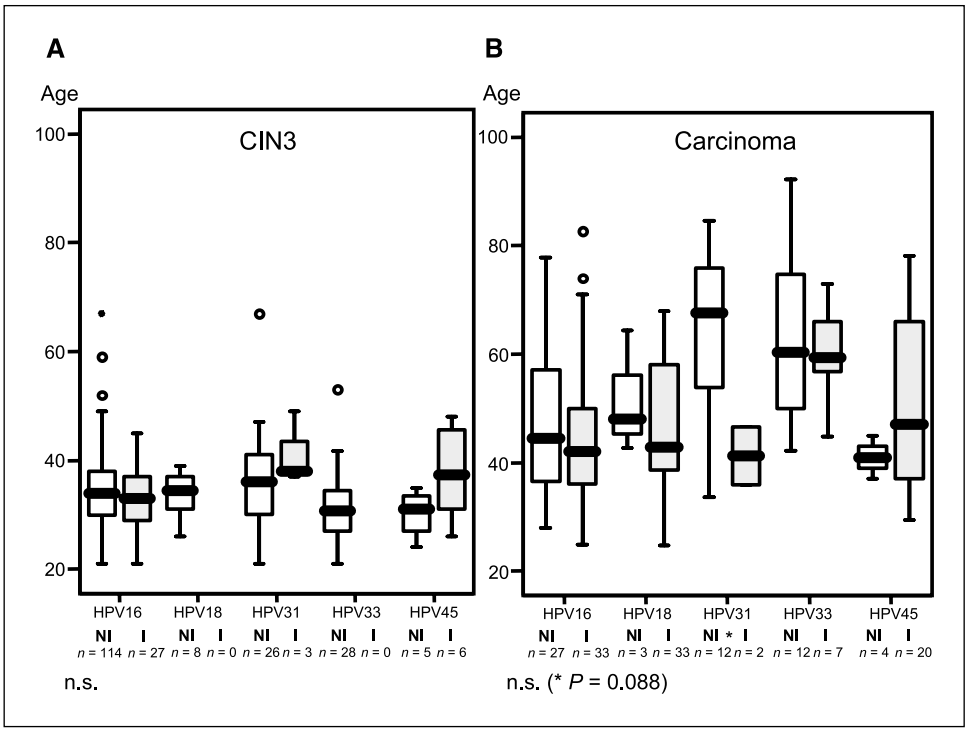

Figure 2. Differences in age at diagnosis with respect to integration status stratified by lesion and HPV type. *A,* box plots of median age at diagnosis of CIN3 by integration status, stratified by HPV type. *Gray boxes,* cases with integration. *B,* box plots of median age at diagnosis of carcinoma by integration status, stratified by HPV type. *Gray boxes,* cases with integration. Median age differences are not significant (Mann-Whitney test). *NI,* nonintegrated; *I,* integrated. *, $P = 0.088$.

showing that integration of HR-HPV genomes may result in disruption of the viral control mechanisms that regulate the expression of papillomavirus genes in their host cells: First, the viral E2 gene that encodes a transcriptional inhibitor of the early HPV promoter is usually disrupted during integration and results in its functional inactivation (22, 35–38); second, cotranscribed cellular sequences may stabilize viral transcripts derived from integrated viral genome copies and, thus, contribute to enhanced viral oncogene expression (24, 25); and third, adjacent cellular sequences may override the control of integrated HPV genomes by its own promoter, resulting in higher levels of viral gene expression even in presence of E2 (23). Thus, HR-HPV integration was hypothesized to trigger the deregulated oncogene expression pattern representing the critical event in cervical carcinogenesis, preceding the development of chromosomal abnormalities that drives malignant progression (39, 40). Several studies using the W12 cell line that was isolated from HPV16-infected keratinocytes and contains exclusively episomal HPV genomes in early passages, but preferentially

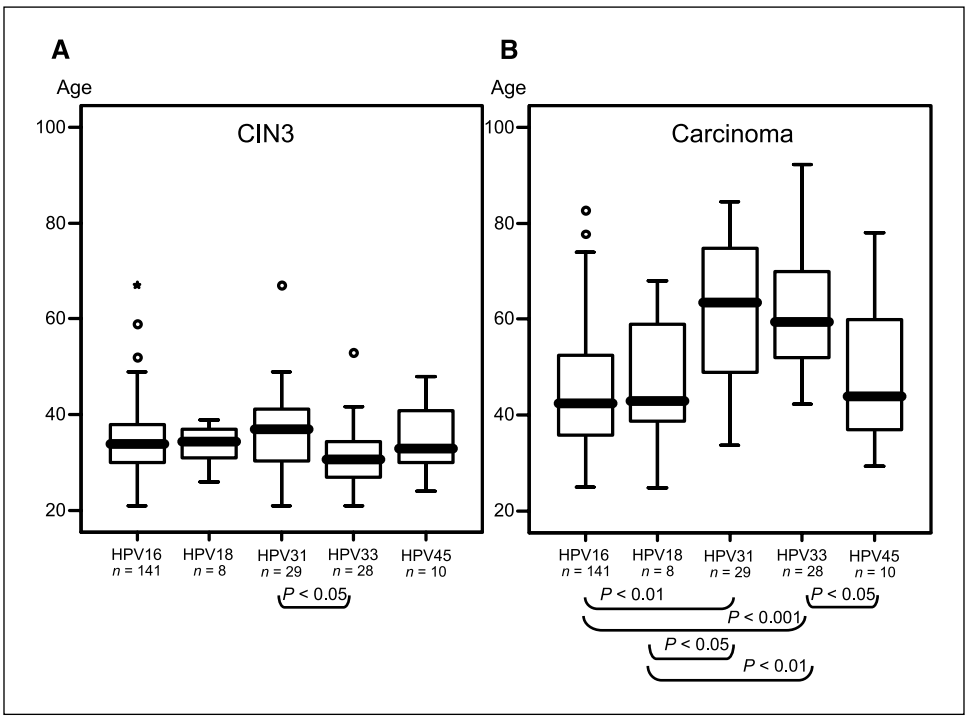

Figure 3. Effect of HPV type on age of diagnosis. *A,* box plots of median age at diagnosis of CIN3 by HPV type, independent of integration status. Differences of median age between HPV31 and HPV33 are significant (Kruskal Wallis test; Dunn's test for multiple comparisons; $P < 0.05$). *B,* box plots of median age at diagnosis of carcinoma by HPV type independent of integration status. Differences of median age between HPV16 and HPV31 ($P < 0.01$), HPV16 and HPV33 ($P < 0.001$), HPV18 and HPV31 ($P < 0.05$), HPV18 and HPV33 ($P < 0.01$), and HPV33 and HPV45 ($P < 0.05$) are significant (Kruskal Wallis test; Dunn's test for multiple comparisons).

integrated HPV genome copies in later passages (41) supported this concept because integration of HPV16 genomes was associated with chromosomal instability and the deregulated expression pattern of the viral oncogenes in this *in vitro* model (17, 42).

However, several studies that have analyzed the prevalence of integrated viral genome copies in invasive cervical carcinoma samples showed that a part of the invasive cancer biopsies did not contain any integrated viral genome copy (15, 21). This suggests that if integration was the major determinant leading to deregulated viral gene expression and transforming infections in cervical carcinogenesis, a substantial part of tumors has to reach this stage via independent pathways. Alternatively, it is conceivable that integration is rather the consequence of chromosomal instability induced by deregulated HR-HPV E6 and E7 expression (43) but not its cause. In that latter scenario, deregulated expression in HR-HPV–infected cells has to be induced by independent pathways. Based on several biomarker studies, it became clear that surrogate markers for HPV oncogene expression such as $p16^{INK4a}$ may be expressed in early dysplastic lesions already (44). However, several studies convincingly show that integration at this early stage of preneoplasia is only exceptionally found (15, 21, 45). Moreover, a direct comparison of the time points for the viral integration and the outgrowth of aneuploid cell clones showed that aneuploidy precedes the outgrowth of cell clones that harbor integrated viral genomes (46). Taken together, these data strongly support the concept that integration of viral genome copies is an important and direct consequence of chromosomal instability that is induced by the deregulated expression of the viral E6 and E7 oncogenes, however, not its cause.

In this study, we aimed to analyze the role of integration with respect to disease progression in a large number of patients by using the APOT assay. This assay was applied to monitor integration of the five most frequent oncogenic HR-HPV types HPV16, 18, 45, 31, and 33. If HPV-integration was an essential prerequisite for malignant progression, we would have expected that the majority of carcinomas, irrespective of the HR-HPV type, display at least one integrated viral genome copy.

Our data clearly confirm the observation that there is an increasing frequency of integrated viral genomes in more advanced preneoplastic lesions. However, only 62% of all carcinoma samples displayed integrated HPV genomes. Moreover, integration of HPV31 and 33 was found substantially less frequent compared with integration of HPV16, 18, and 45.

Based on the low integration frequencies detected for the HPV31 and 33 types that were previously described to be less aggressive compared with HPV16, 18, and 45, we speculated whether cases with integrated HPV genomes might progress to carcinomas faster, and thus, the median age at diagnosis for cases with integration would be lower. We did not observe any clear effect of integration status on median age at diagnosis of cancer and tumor stage. However, there was a strong effect on the underlying HPV type on median age of the patients. Taken together, we found an association between the integration frequency of specific HPV types and the median age at diagnosis of cancer (Fig. 4).

If one assumes that the integration of the viral genome is a direct consequence of the degree of chromosomal instability induced by the viral oncogenes expressed in replicating basal epithelial cells, the data described in this study suggest that the oncogenes of HPV16, 18, and 45 confer substantially more chromosomal instability compared with the oncogenes of HPV31 and 33. This hypothesis is supported by two major findings of this study:

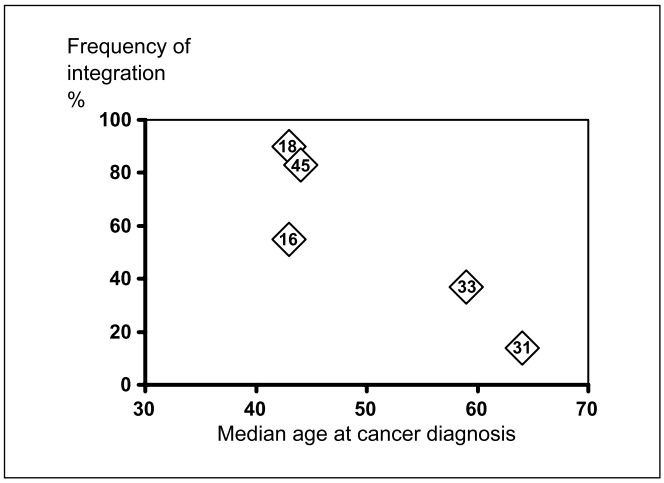

Figure 4. Correlation between integration frequency and age. Schematic presentation of relation between age at diagnosis of cancer and integration frequency by HPV type. Closely related types HPV18 and HPV45 show similar characteristics. HPV31 and HPV33 show both a high age at diagnosis and a low integration frequency. HPV16 is intermediate.

(*a*) integration of HPV16, 18, and 45 genomes is observed more frequently than integration of HPV31 and 33 in invasive cancers and (*b*) all high-grade precancers (CIN 3 lesions) occur at a similar age independent of the respective HR-HPV type that was responsible for the outgrowth of the lesion. However, marked differences were observed in the median age of patients that developed cancers associated with the same HR-HPV types. This suggests that once the deregulated expression pattern of the viral oncogenes occurred (at latest once the lesions reached the stage of a CIN 2/3 lesion) HPV31 or 33 associated lesions required substantially more time to progress to invasive carcinomas compared with lesions induced by the apparently more potent oncogenes of HPV16 and 18. This finding is in good agreement with recent reports that suggest that patients infected with HPV16 and 18 are at a substantially higher risk of progression to high-grade disease compared with patients infected with non-16 and non-18 HR-HPV genotypes (7, 8). It is further in good agreement with the observation that cancers associated with HPV16 and HPV18 tend to be more aggressive, and that the detection of HPV types other than 16 and 18 is an independent predictor of better survival in patients with cervical carcinoma (47–50).

In conclusion, our data strongly suggest that HPV integration is not an essential event in cervical carcinogenesis. The integration status itself was not found to be associated with younger age at diagnosis of cancer. In contrast, the underlying HPV type had a strong effect, and the integration frequency of various HPV types correlated strongly with the age at diagnosis of cancer, suggesting that the malignant potential of the various HR-HPV types is reflected by their integration frequency in invasive cervical carcinomas.

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