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Systematic Review of Genomic Integration Sites of Human Papillomavirus Genomes in Epithelial Dysplasia and Invasive Cancer of the Female Lower Genital Tract

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ABSTRACT

Cancers of the anogenital tract as well as some head and neck cancers are caused by persistent infections with high-risk type human papillomaviruses (HPVs). Two viral oncogenes, E6 and E7, induce severe chromosomal instability associated with centrosome aberrations, anaphase bridges, chromosome lagging, and breaking. This occurs early in preneoplastic lesions, when the viral genome still persists in an episomal state. In most invasive cancers and also in a few high-grade dysplastic lesions, however, integration of high-risk HPV genomes into the host genome is observed. Integration seems to be a direct consequence of chromosomal instability and an important molecular event in the progression of preneoplastic lesions. Disruption or deregulation of defined critical cellular gene functions by *insertional mutagenesis* by integrated HPV genome fragments has been hypothesized as one major promoting factor in the pathogenesis of HPV-associated cancers. This hypothesis was based on the detection of HPV integration events in the area of tumor-relevant genes in few cases. Here, we reviewed >190 reported integration loci with respect to changes in the viral structure and the targeted genomic locus. This analysis confirms that HPV integration sites are randomly distributed over the whole genome with a clear predilection for genomic fragile sites. No evidence for targeted disruption or functional alteration of critical cellular genes by the integrated viral sequences could be found.

INTRODUCTION

Persistent infections with high-risk (HR) human papillomaviruses (HPVs), *e.g.,* HPV-16, HPV-18, HPV-31, HPV-33, and HPV-45 have been identified as an essential although not sufficient factor in the pathogenesis of anogenital and other epithelial carcinomas (1) HR-HPV genomes encode two proteins, E6 and E7, that interfere with important cellular control mechanisms of the cell cycle, apoptosis, and maintenance of chromosomal stability. The effects of E6 and E7 on p53 and pRB as well as on many other cellular proteins have been extensively investigated in the past and significant alterations of the regulation of the cell cycle could be attributed to the biochemical interaction of the two viral oncogenes to their respective cellular binding partners (2, 3). Moreover, recent studies demonstrated that the two viral oncoproteins cooperatively disturb the mechanisms of chromosome duplication and segregation during mitosis and induce thereby severe chromosomal instability (4).

HR-HPV genomes replicate as episomal molecules in the normal viral life cycle. Although the HPV genome is consistently retained in an episomal state in early dysplastic low-grade lesions, the whole viral genome or fragments thereof are covalently integrated into the chromosomal DNA of the host cell in some advanced HPV-associated precancers and the majority of HR-HPV-associated carcinomas (5–8). These observations suggest that integration of viral genes in severe dysplastic lesions strongly enhance neoplastic progression to invasive carcinomas. A possible reason for the progression toward malignant lesions after HR-HPV integration might be structural changes of the viral genome that allow enhanced and deregulated expression of the viral oncogenes and thereby confer the additional neoplastic selective pressure. In addition to that, it has also been speculated that critical cellular genes are affected by integration of viral genome fragments and that interference of viral sequences with critical cellular sequences contributes essentially to the enhanced progression risk of HPV-induced preneoplasia into neoplastic lesions (9–12).

It was shown that HPV E6- and E7-encoding cDNAs derived from integrated viral oncogene transcripts confer a much stronger transforming capacity in primary cells as compared with cDNAs derived from episome-derived transcripts. This was attributed to the longer half-life of transcripts derived from integrated HPV DNA, mediated by 3′-cellular sequences of the fusion transcripts (13). The relative expression levels of the viral oncogenes and their corresponding gene products appear to be directly influenced by the sequence context of individual integration sites. In addition, *cis*-acting regulatory sequences were shown to exert a strong influence on the expression level and regulation of the integrated viral oncogenes (14). Additional work demonstrated that in specific cervical cancer cell lines only one or few integrated genomes are transcribed, whereas many others within the same cells are transcriptionally silenced (15). In contrast, clinical samples harbor only few integration sites, with the majority thereof being actively transcribed (16). Taken together, these observations suggest that integration of the viral genome renders viral gene expression independent of viral control mechanisms and allows selection of cell clones with deregulated viral oncogene expression favoring the outgrowth of neoplastic cell clones. Thus, the current evidence clearly points to an important impact of cellular sequences on the integrated viral genomes; however, it remains unclear whether the influence of viral sequences on defined cellular genes similarly contributes to the progression of HPV-induced dysplasia. Various murine, avian, and feline retroviruses can transform cells either by affecting the regulation and/or disrupting the structure of tumor suppressing or oncogenic cellular genes (transforming retroviruses; Ref. 17). This process of *insertional mutagenesis* is random, usually affects many different genomic loci, and is highly inefficient with regard to the transformation efficacy of single infection events, features that seem to be shared by oncogenic HPVs. In some cases, HPV integration has been found to occur in or close to potentially tumor relevant genes, especially within or close to the *MYC* gene locus (9, 10, 18).

Schwarz *et al.* (19) identified an integrated genome copy of HPV-68 in the cervical carcinoma cell line ME180. The viral genome disrupts one allele of a novel tumor suppressor gene, *APM-1*. The nonaffected allele was lost in these cells, suggesting that lack of *APM-1* function contributed to the pathogenesis of this particular cancer cell clone. In a recent report, Ferber *et al.* (11) described three cervical carcinoma cases in which integration was observed in the area of the *telomerase gene*. Strong up-regulation of *hTERT* expression was observed in one of these samples.

Many different assays have been applied to analyze genomic HPV integration sites. *In situ* hybridization using HPV-specific probes has given a rough estimate about the distribution of integrated HPV genomes in cell lines. Although an accumulation of integrated HPV genomes was observed at few loci, a general integration hot spot could

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not be identified (12, 20–22). Several PCR-based protocols have been developed that enable the analysis of HPV integration in clinical samples at larger scales. Direct methods to monitor integrated HPV genome copies imply that the HPV sequences are coamplified together with flanking cellular sequences using either enzyme digestion and adaptor ligation [detection of integrated papillomavirus sequences (DIPS) by ligation-mediated-PCR (23)] or religation followed by inverse PCR (24). In another protocol, fusion regions are amplified using HPV-specific primers and primers that bind to distinct restriction enzyme recognition sites [restriction-PCR (25)]. An additional method was referred to as amplification of papillomavirus oncogene transcripts (APOT) assay (7). Here, a modified 3′-rapid amplification of cDNA ends PCR using upstream HPV E7-specific and downstream oligo dT adaptor primers were applied to amplify HPV E7-specific transcripts either derived from integrated or episomal viral genome copies.

Up to now, in total, 192 individual HPV integration sites have been described in primary tumor samples and cell lines. Here, we summarize all available data on chromosomal HPV integration sites. The data suggest that integration of HR-HPV genomes occurs relatively late in the progression of high-grade cervical dysplasia. It appears that integration of HR-HPV genomes is a consequence of an overall destabilization process of the chromosomal integrity in replicating epithelial stem cells that express the viral E6-E7 oncogenes. The consequences of the structural alterations of the viral genome and the impact of cellular sequences on its transcriptional regulation seem to be more important than functional alteration of specific cellular genes by the integrated viral sequences.

Table 1 *Publications included in this study*

Author	Method	Number and type of integrate-positive samples analyzed
Mincheva *et al.* (22)	FISH[a]	*Hela, Caski, SiHa*[b]
Canizzaro *et al.* (20)	FISH	*C4-I*, 1 CxCa
Parton *et al.* (40)	FISH	*SVD2*
Couturier *et al.* (41)	FISH	*IC1, IC2, IC3, IC4*
Hori *et al.* (42)	FISH	cell line
Smith *et al.* (43)	FISH	6 cell lines
Gilles *et al.* (44)	FISH	*CK1, CK11*
Koopman *et al.* (21)	FISH	*CSCC-1, CSCC-7, CC-8, CC-10A/B, CC-11*
Durst *et al.* (9)	Genomic library	*SiHa, SW756, C4-1, HeLa, 1 CxCA*
Wagatsuma *et al.* (45)	Genomic library	4 CxCa
Kahn *et al.* (46)	Genomic library	1 tonsillar carcinoma
Choo *et al.* (47)	HPV-random PCR	*Caski*, 4 CxCa
Reuter *et al.* (19)	mRNA library	*ME180*
Thorland *et al.* (25)	Restriction PCR	7 CxCa
Kalantari *et al.* (24)	Inverse PCR	5 CxCa
Luft *et al.* (23)	DIPS	17 CxCa, 1 VIN3
Shera *et al.* (48)	ALU PCR	5 CxCa
Einstein *et al.* (49)	Genomic library	1 CxCa
Peitsaro *et al.* (50)	APOT	*UT-DEC1*
Wentzensen *et al.* (18)	APOT	55 CIN2-3, CxCa, VIN, VaIN
Wiest *et al.* (51)	APOT	2 head and neck cancers
Thorland *et al.* (52)	Restriction PCR	17 CxCa
Ziegert *et al.* (16)	DIPS/APOT	22 CIN3 and CxCa
Ferber *et al.* (11)	Restriction PCR	3 CxCa
Ferber *et al.* (10)	Restriction PCR	18 CxCa

[a] FISH, fluorescence *in situ* hybridization; HPV, human papillomavirus; DIPS, detection of integrated papillomavirus sequences; APOT, amplification of papilloma virus oncogene transcripts.
[b] italics: cell lines, CxCa, cervical carcinoma; CIN, cervical intraepithelial neoplasia; VaIN, vaginal intraepithelial neoplasia; VIN, vulvar intraepithelial neoplasia.

MATERIALS AND METHODS

Data Collection. To collect data on chromosomal loci that are affected by integration of HR-HPV genomes, an extensive PubMed search was performed. All articles were included that presented data on either chromosomal localizations or exact nucleotide sequences of integration sites or viral cellular fusion regions. Several larger clinical studies have only looked at the HPV integration status but did neither provide locus nor sequence information (5–8). These studies were not included in the analysis

Methods used to detect chromosomal loci hit by integration of HR-HPV genomes cover methods such as fluorescence *in situ* hybridization and genomic and RNA library techniques to PCR-based amplification of viral cellular genomic fusions or fusion transcripts. Only integration events that could be clearly mapped to a specific human sequence were included in the study, thereby omitting many integration events into repetitive genomic areas.

Database Analysis of Integration Sites. Where sequence data were available, a BLASTN (26) database comparison of cellular sequences with the most recent update of the human genome sequence was performed.[1] All mapping data were updated when necessary. Several integration sites were reassigned to different chromosomal bandings than those initially described.

Integration Database Internet Resource. Table 2 is available as a continuously updated integration database.[2]

RESULTS AND DISCUSSION

Overview of Studies and Samples. In total, 25 studies were included in the analysis covering 192 individual integration events (Table 1). Eight studies used fluorescence *in situ* hybridization to map integration sites, the remaining 17 studies used different PCR-based protocols to generate sequence information of the respective loci. Twenty-one integration sites were derived from cell lines, 171 from clinical samples. The majority of integration events (157) has been described for cervical lesions, mainly cervical carcinomas, but also for CIN3 (9) and CIN2 (1) lesions. Six published integration sites were discovered in vulvar, 4 in vaginal lesions, and 1 in a penile carcinoma.

Furthermore, HPV integration loci of three head and neck as well as tonsillar cancer samples have been published. The prevailing HPV type in the studies is HPV-16 with 119 integrations, followed by HPV-18 (64), HPV-45 (3), HPV-33 (2), HPV-6a, HPV-1, HPV-67, and HPV-68 (each one). However, these numbers do not reflect the real distribution of HR-HPV in the respective lesions because most PCR-based methods have only been established for HPV-16 and HPV-18.

Twenty-three integration sites were mapped with *in situ* hybridization and 169 localizations derived from PCR-based methods providing sequence information; some of these samples have additionally been analyzed with fluorescence *in situ* hybridization techniques. The most frequently used PCR-based methods were the amplification of papillomavirus oncogene transcripts (APOT) assay (57), the restriction PCR method (47) and the DIPS assay (32), accounting for 136 mapped integration sites. Apart from that, PCR techniques involving enzyme digestion and religation (inverse PCR), ALU-PCR, and randomly primed PCR were used on a small number of samples as well as genomic and mRNA library techniques (Table 2).

Sequences at Integration Sites. Several studies have looked at the exact integration site and analyzed the transition sequence between viral and cellular genome. With respect to the nucleotide sequence, all integration sites are different. Neither a specific cellular sequence motif has been observed, nor have recurrent integrations in a specific area happened at the similar nucleotides. Likewise, there is no constant disruption site in the viral genome, and the transitions from viral to cellular sequences can be found anywhere from early E1 to the late genes. Homologous recombination involving larger areas of similar nucleotide sequences does not seem to play a role in HPV integration. Geisen *et al.* (27) have described a human sequence that shows a mild

[1] Internet address: http://www.ncbi.nlm.nih.gov/BLAST/.
[2] Internet address: http://www.med.uni-heidelberg.de/patho/pathomol/AG_onkogene_Papillomvirusinfektionen.html.

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Table 2 *Summary of all published HPV integration sites*

Name	Pathology	Human papillomavirus	Locus	Fragile site[a]	Gene[b]	Detection method	Reference no.	Fusion region[c]
int9	Ca[d]	16	1p36	FRA1A	WASF2	APOT	18	
V18	CA (Sq)	16	1p34		BMP8	Random PCR	47	3′ Ont
FEH18L	CL	18	1p22–31			FISH	43	
FEA	CL	18	1q12–q21			FISH	43	
CC192	Ca (Sq)	16	1q21	FRA1F	HS8015	Restriction PCR	25	
int73	Ca	16	1q21		RPS27	APOT	18	
CC10	CL	45	1q21–23			FISH	21	
F826	Ca	16	1q25			Inverse PCR	24	3′ Ont
T5	Ca	16	1q25			DIPS	23	
MC360	Ca	18	1q31	FRA1K		Restriction PCR	11	
int69	CIN3	16	1q32		HS205126	APOT	18	
LU7	Ca	18	1q32.2	FRA1K	CD34	Restriction PCR	11	
102	Ca	16	1q41	FRA1H		Restriction PCR	52	
T17	Ca	16	1q41			DIPS	23	3′ 0nt
T32	Ca	18	1q42		HS285861	APOT/DIPS	16	3′ 1nt
int66	Ca	18	1q43		CHS1	APOT	18	
T3	Ca	16	2			DIPS	23	
T4	Ca	16	2			DIPS	23	
IC4	CL	18	2p24			FISH	41	
int3	Ca	16	2p24			APOT	18	
T19	Ca	16	2q21		LRP1B	APOT/DIPS	16	
T17	Ca	16	2q22			APOT/DIPS	16	3′ 2nt
T1	Ca	16	2q22			DIPS	23	
HK9	Ca	18	2q22.1	FRA2F	LRP1B	Restriction PCR	11	
int70	Ca	16	2q23	FRA2K		APOT	18	
int29	Ca	16	2q24	FRA2K	NR4A2	APOT	18	
int52	CIN3	16	2q31	FRA2G		APOT	18	
int71	Ca	16	2q32	FRA2H	GLS	APOT	18	
AC-8	Ca	18	2q32			ALU PCR	48	3′ filler 20nt
int45	Ca	16	2q33	FRA2I	AA084805	APOT	18	
SVD2	CL	1	2q33			FISH/mRNA lib	40	
H404	Ca	16	2q33			Genomic library	45	
T04	CIN3	16	2q34		HS25235	DIPS	16	3′ 2nt
M15	Ca (Sq)	16	2q34–35		MAP-2	Random PCR	47	5′ filler 10nt 3′ 1nt
CC-6	Ca	18	2q36			ALU PCR	48	3′ 3nt
1.2a (probe)	Ca	16	3p25				9	
CC-6	Ca	18	3p25			ALU PCR	48	
CC11	CL	67	t(3;13)(p23–26;q22–331)			FISH	21	
n.n.	CL	18	3p21–22			FISH	42	
int65	Ca	18	3p21			APOT	18	
T14	Ca	16	3p21		MAP4	APOT/DIPS	16	3′ 2nt
H705	Ca (Sq)	16	3p14	FRA3B		Genomic library	45	
61	Ca	16	3p14	FRA3B		Restriction PCR	52	
MC123	Ca	18	3p14	FRA3B	FHIT	Restriction PCR	11	
int72	Ca	16	3q21	FRA3F	AI555655	APOT	18	
CC8	CL	45	3q26–29			FISH	21	
T31	Ca	16	3q27			APOT/DIPS	16	
T05	CIN3	16	3q27			DIPS	16	3′ 2nt
F3155	Ca	16	3q28			Inverse PCR	24	5′ 1nt
int32	VIN3	16	3q28	FRA3C		APOT	18	
int4	VINX	16	3q28	FRA3C	TP63	APOT	18	
int26	Ca	18	3q28	FRA3C		APOT	18	
int2	Ca	16	4p16	FRA4A		APOT	18	
T13	Ca	18	4p15		PCDH7	APOT/DIPS	16	3′ 2nt
T28	VaCa	16	4q21		BIKE	DIPS	16	3′ 6nt
int35	Ca	18	4q21			APOT	18	
int68	CIN3	16	4q21		PTPN13	APOT	18	
int18	VIN2	16	4q31		FRA4C	APOT	18	
61	Ca	16	5p15.3			Restriction PCR	52	
MC11	Ca	18	5p15		HTERT	Restriction PCR	10	
HK1	Ca	18	5p15		HTERT	Restriction PCR	10	
HK2	Ca	18	5p15		HTERT	Restriction PCR	10	
MC315	Ca	18	5p13.2	FRA5E	SLC1A3	Restriction PCR	11	
HeLa	CL	18	5p11–15			FISH/ISH	12 9 20	
UT-DEC-1	CL	33	5p14	FRA5E		APOT, genomic	50	3′ filler 16nt
T3	HNSCC	16	5q14	FRA5E		APOT	51	
HK16	Ca	18	5q15	FRA5D		Restriction PCR	11	
int46	Ca	16	5q31	FRA5C	KLHL3	APOT	18	
86	Ca	16	5q31	FRA5C	FLJ23312	Restriction PCR	52	
24	Ca	16	5q35		KCNIP1	Restriction PCR	52	
int17	Ca	16	5q35	FRA5G		APOT	18	
int23	CIN3	16	6p			APOT	18	
F3165	Ca	16	6p25			Inverse PCR	24	5′ 4nt
T8	HNSCC	16	6p24			APOT	51	
CC-2	Ca	18	6p24			ALU PCR	48	3′ filler 17nt
T18	Ca	16	6p24		HS171942	APOT/DIPS	16	3′ 2nt
T21	Ca	18	6p23		HS633327	APOT/DIPS	16	3′ 0nt
CC171	Ca (Sq)	16	6p22	FRA6C		Restriction PCR	25	

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Table 2 *Continued*

Name	Pathology	Human papillomavirus	Locus	Fragile site[a]	Gene[b]	Detection method	Reference no.	Fusion region[c]
H901	Ca	16	6p21.3			Genomic library	45	5′ 3nt 3′ 1nt
int76	Ca	16	6q21	FRA6F		APOT	18	
191	Ca	16	6q21	FRA6F		Restriction PCR	52	
int21	VIN3	16	6q25	FRA6E	TCP1	APOT	18	
MC123	Ca	18	6q26	FRA6E	KIAA1838	Restriction PCR	11	
T25	Ca	16	7p22	FRA7B	DGKB	DIPS	16	
V15	Ca	16	7p22	FRA7B		Random PCR	47	5′ 1nt 3′ 0nt
T6	Ca	18	7q31		FOXP2	DIPS	23	3′ filler 6nt
MC315	Ca	18	7q31	FRA7G		Restriction PCR	11	
T13	Ca	16	7q31			DIPS	23	
int42	Ca	16	8p23		HS162183	APOT	18	
MC123	Ca	18	8p21		EPHX2	Restriction PCR	11	
CC5a	Ca (Sq)	16	8p12			Random PCR	47	3′ 1nt
int16	Ca	18	8p11.2		HS127775	DIPS, APOT	23 18	3′ 5nt
C4-1	CL	18	8q21–22	FRA8B		FISH, APOT	22 18	
61	Ca	16	8q21.3	FRA8B	RIPK2	Restriction PCR	52	
T29	Ca	18	8q24		FLJ10359	APOT/DIPS	16	3′ 1nt
IC1	Ca	18	8q24			FISH	41	
IC2	Penile Ca	16	8q24.1			FISH	41	
IC3	Ca	16	8q24.1			FISH	41	
MC31	Ca	18	8q24	FRA8C	MYC	Restriction PCR	11	
MC391	Ca	18	8q24	FRA8C	MYC	Restriction PCR	11	
MC398	Ca	18	8q24	FRA8C	MYC	Restriction PCR	11	
LU8	Ca	18	8q24	FRA8C	MYC	Restriction PCR	11	
HK10	Ca	18	8q24	FRA8C	MYC	Restriction PCR	11	
HeLa	CL	18	8q24	FRA8C	MYC	FISH, APOT	12 9 20 18 11	
int41	Ca	16	8q24	FRA8C		APOT	18	
int25	Ca	18	8q24	FRA8C	MYC	APOT	18	
MC415	Ca	18	8q24.2	FRA8C/D	MYC	Restriction PCR	11	
94	Ca	16	9p24.1			Restriction PCR	52	
T23	Ca	18	9p22		NFIB	APOT/DIPS	16	3′ 0nt
int44	Ca	16	9p13			APOT	18	
CK11	CL	33	9p13			FISH	44	
int5	Ca	16	9q22	FRA9D	FANCC	APOT	18	
HK13	Ca	18	9q22	FRA9D	FANCC	Restriction PCR	11	
HeLa	CL	18	9q31–34			FISH	9 20	
T22	Ca	18	9q34		HS323813	DIPS	6	3′ 2nt
317	Ca	16	9q34		Notch1	Restriction PCR	52	
int36	Ca	16	9q34			APOT	18	
V1	VIN3	16	10p15			DIPS	23	3′ 2nt
int19	Ca	16	10p15			APOT	18	
int79	Ca	16	10q22	FRA10D		APOT	18	
75	Ca	16	10q22	FRA10D		Restriction PCR	52	
int78	Ca	16	10q23	FRA10A	FER1L3	APOT	18	
n.n.	Tonsillar Ca	6a	10q24			genomic library	46	
int34	CIN2	16	10q26	FRA10F		APOT	18	
73	Ca	16	11p15.5		MUC5B	Restriction PCR	52	
F338	Ca	16	11p13			Inverse PCR	24	5′ 4nt 3′ 3nt
T15	Ca	16	11q14			DIPS	23	3′ 3nt
SW756	CL	18	12q13	FRA12A		FISH	12 11	
SKv	CL	16	12q13			FISH	53	
73	Ca	16	12q13			Restriction PCR	52	
int27	Ca	18	12q21		ALU	APOT	18	
61	Ca	16	12q21.31		MYF5	Restriction PCR	52	
327	Ca	16	12q23.1		ELK3	Restriction PCR	52	
TC146	CL	16	13q14			FISH	54	
int50	VIN3	16	13q14		KPNA3	APOT	18	
SiHa	CL	16	13q21	FRA13B	ALU	FISH, APOT	18 18	
int67	CIN3	16	13q21	FRA13B		APOT	18	
int30	Ca	16	13q21	FRA13B		APOT	18	
int58	Ca	16	13q21	FRA13B		APOT	18	
int10	Ca	16	13q21.1	FRA13B		APOT	18	
int57	CIN3	16	13q22			APOT	18	
80	Ca	16	13q22.2	FRA13C		Restriction PCR	52	
84	Ca	16	13q22.2	FRA13C		Restriction PCR	52	
CK1	CL	33	13q33–34			FISH	44	
T11	Ca	18	14			DIPS	23	
CSCC7	Ca	16	t(3;14)(p14.1 14.3;14)			FISH	21	
CSCC1	Ca	16	14q			FISH	21	
int55	Ca	18	14q13			APOT	18	
int1	Ca	16	14q24.1	FRA14C	HS57811	APOT	18	
HK15	Ca	18	14q24.1	FRA14C		Restriction PCR	11	

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Table 2 *Continued*

Name	Pathology	Human papillomavirus	Locus	Fragile site[a]	Gene[b]	Detection method	Reference no.	Fusion region[c]
T7	Ca	16	14q24.2			DIPS	23	
T15	Ca	16	14q24.3			DIPS	23	3′ 0nt
MC34	Ca	18	14q24	FRA14C	RNGTT	Restriction PCR	11	
T8	Ca	16	14q32			DIPS	23	
HKcHPV 16d-2	CL	16	14q32			FISH	12	
n.n.	Ca	16	14q32.3		TNFAIP2	genomic lib.	49	duplication 5′ 11nt, 3′ 5nt
int14	ValN3	18	15q12		HS150715	APOT	18	
int62	Ca	16	15q15		HS195730	APOT	18	
91	Ca	16	15q15			Restriction PCR	52	
LU3	Ca	18	15q23	FRA15A		Restriction PCR	11	
T3	Ca	16	16			DIPS	23	
CC-4	Ca	18	16p12			ALU PCR	48	3′ 3nt
int61	Ca	16	16q22	FRA16B	AFP	APOT	18	
T15	Ca	16	16q24		CDH13	APOT/DIPS	16	3′ filler 9nt
H022	Ca	16	17q11			Genomic library	45	5′ 6nt 3′ 5nt
int33	Ca	16	17q12		HS23106	APOT	18	
AC-8	Ca	18	17q21		ERBB2	ALU PCR	48	3′ filler 11nt
T16	Ca	18	17q23.2	FRA17B		DIPS	23	3′ 3nt
T30	Ca	18	17q23			APOT/DIPS	16	3′ filler 4nt
T24	Ca	18	17q23			DIPS	16	3′ 3nt
T26	Ca	18	17q23		HS355936	APOT/DIPS	16	
CC226	Ca	16	17q23	FRA17B		Restriction PCR	25	
int13	Ca	18	17q23	FRA17B	HS12677	APOT	18	
107	Ca	16	17q23.2	FRA17B	DKFZP566I133	Restriction PCR	52	
207	Ca	16	17q23.2	FRA17B		Restriction PCR	52	
T9	Ca	16	17q25			DIPS	23	3′ 2nt
ME180	CL	68	18q21		APM-1	mRNA library	19	
F2423	Ca	16	18q22			Inverse PCR	24	5′ 5nt 3′ 2nt
HK11	Ca	18	19p13		PRKACA	Restriction PCR	11	
LU2	Ca	18	19q13	FRA19A	CEACAM5	Restriction PCR	11	
int54	CIN3	16	19q13	FRA19A	CEACAM5	APOT	18	
265	Ca	16	20p12			Restriction PCR	52	
int8	Ca	16	20p12	FRA20B		APOT	18	
int6	Ca	16	20p11	FRA20A	HS97790	APOT	18	
3.2a (probe)	CA	16	20q13				9	
int15	Ca	18	21q21			APOT	18	
int20	ValN2	16	21q22			APOT	18	
HeLa	CL	18	22q12–13			FISH	12 9 20	
CC10	CL	45	22q13			FISH	21	
T27	VaCa	16	Xp22		HSX11910	APOT/DIPS	16	

[a] Fragile site correlates with integration locus, either by direct visualization or by database comparison.
[b] Known gene or Unigene Cluster that correlates with integration locus.
[c] Fusion region 5′ and 3′: xnt, identity between viral and cellular sequence; filler, sequence that neither derives from viral nor cellular sequence at that locus.
[d] Ca, cervical carcinoma; APOT, amplification of papilloma virus oncogene transcripts; Sq, squamous cell; CL, cell line; FISH, fluorescence *in situ* hybridization; DIPS, detection of integrated papillomavirus sequences; CIN, cervical intraepithelial neoplasia; VIN, vulvar intraepithelial neoplasia; VaCa, vaginal carcinoma; HNSCC, head and neck squamous cell cancer; VaIN, vaginal intraepithelial neoplasia.

degree of similarity to HPV E5 located on chromosome 7p13, but it has thus far not been reported to be a HPV integration target.

A sequence analysis of the fusion region between the viral and the cellular genome was possible in 40 cases (Table 2). Seven integrated HPV genomes were characterized on both fusion sides; for the remaining 33, only sequences from either the 5′- or the 3′-fusion region were available. In 27 cases, short overlapping sequences between one and six nucleotides could be found. Six samples showed a direct transition from viral to cellular sequence, and in seven other cases, filler sequences were found at the fusion site that neither derived from viral nor cellular sequences at the respective locus. Short identities in the fusion region seem to facilitate integration. In some cases, major chromosomal changes must have occurred upon integration, probably involving DNA loops that lead to the transfer of distant sequences to the integration site.

Deregulation of Cellular Genes by HPV Integration. In several cases, HPV integration has occurred in or close to known genes, most frequently in intronic regions. Although coding regions are only rarely hit by HPV, gene expression and mRNA structure can be severely altered by insertion of the strong HPV promoter as well as additional splice donor and acceptor sites located on the HPV genome. The expression analyses of integrated HPV DNA have shown that transcribed and coding regions of genes are frequently cotranscribed with HPV E6 and E7 oncogenes.

Some of the genes disrupted by HPV integration are known to be involved in tumor development in other cancer entities, *e.g., MYC, TP63, NR4A2, APM-1, FANCC, TNFAIP2,* and *hTERT*. However, only few examples exist where a direct link between HPV integration and gene alteration was shown by functional data. In the case of the cell line ME180, HPV-68 integration was found in a novel tumor suppressor gene, *APM-1* (19). It could be shown that the unaffected allele was lost in that cell line and that *APM-1* levels were reduced as compared with other cell lines. Transfection of HeLa and Caski cell lines with *APM-1* led to reduced growth rates in colony-forming assays. Repeated integration in the area of a specific tumor relevant gene is rare; accumulation of integrated HPVs has been found in the greater area of the *MYC* locus; apart from that, integration in or close to *FANCC, hTERT,* and *CEACAM5* has been described in two or three independent samples from different studies (10, 11, 18).

The highest number of integration events was observed at 8q24, a large chromosome banding that covers ~30 Mb and, among others, harbors the *MYC* gene. 8q24 integration was observed in 12 clinical samples and the cervical cancer cell line HeLa (9, 10, 18). The integration sites are distributed over >500 kb around the *MYC* gene.

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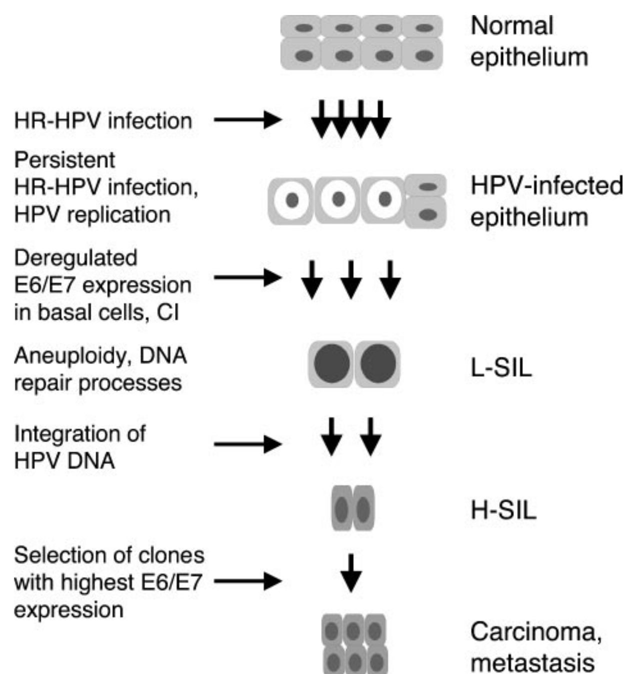

Fig. 1. Role of human papillomavirus (HPV) integration in the progression from normal epithelium to invasive carcinoma. L-SIL, low-grade squamous intraepithelial lesion; H-SIL, high-grade squamous intraepithelial lesion.

Thus far, *MYC* expression analyses of the clinical samples that showed HPV integration in the *MYC* area have not been published. For HeLa, increased MYC-RNA levels were demonstrated by Northern blotting (9). We have previously isolated a fusion transcript from HeLa encompassing viral and cellular sequences derived from the 5′-noncoding *MYC* region (18). However, in contrast to Burkitt's lymphoma, where structural aberrations of the *MYC* locus were clearly shown to be associated with the induction and maintenance of a malignant phenotype, this has not been demonstrated for HPV-induced carcinogenesis.

For *FANCC*, a transcript was identified in a clinical sample that showed the FANCC exon 6 fused to the HPV E6/E7 sequences (18). However, there is no functional evidence that integration at this locus has a major impact on the transformation process. One of three cases with integration in the *hTERT* locus showed indeed strong up-regulation of hTERT transcription (11). Albeit, given the frequently observed telomerase activation in cervical cancer independent of the integration status, one cannot exclude a coincidence of telomerase activation and HPV integration at that locus.

Taken together, this comprehensive set of data does not support the hypothesis that targeted modification of critical cellular genes plays a major role in the progression of HPV-induced preneoplasia. In contrast to the well-documented impact of E6 and E7 expression for HPV-induced transformation (28–33), it has not been shown in a single case that the malignant phenotype of cells relies on the potentially critical changes induced by HPV integration.

Integration of HPV DNA in Fragile Sites. Fragile sites are genomic regions prone to chromosome breaks that facilitate foreign DNA integration. Although some specific sequence motifs were identified for rare fragile sites, common fragile sites do not seem to be linked to a specific genomic sequence and span very large genomic areas (34)

Looking at a larger scale, there seems to be an equal distribution of HPV integration sites in the human genome. All chromosomes were found to harbor integrated HPV genomes at various chromosomal bandings; however, several weakly preferred chromosomal regions were recognized, including 1q21, 2q22, 2q33, 3p21, 3p14, 3q28, 4q21, 5p15, 6p24, 8q24, 9q34, 12q13, 13q21-22, 14q24, and 17q23. They all encompass known fragile sites except for 3p21, 4q21, 5p15, 6p24, and 9q34.

Some studies have directly visualized the coincidence of fragile sites and HPV integration site (10, 25, 35). In other studies, exact chromosomal localizations were compared with mapped fragile sites in the database (18). Here, we reanalyzed all published loci for mapped fragile sites. A limitation of this approach is the rather imprecise mapping of fragile sites. Taking all data together, there is a high correlation between fragile sites and HPV integration sites. In 38% of the 192 integration sites, fragile sites are hit by HPV integration, and the number is probably much higher because some studies did not provide sufficient sequence information and not all of the fragile sites have been mapped thus far. Ten of 15 regions with at least three independent HPV integration events harbor known fragile sites, including the frequently targeted *MYC* locus (8q24). Matzner *et al.* (36) have analyzed integration of vector DNA containing a multidrug resistance gene in a breast cancer cell line under chemotherapy treatment. Here, cell clones grow out after random integration because an external gene confers the drug resistance. A significant overlap of multidrug resistance integration sites, fragile sites, and the clustered HPV integration sites (1p36, 1q21, 6q21, 9q34, 12q13 and 13q22) reviewed here was observed. In total, 62 of the 192 HPV integration loci correlated with the multidrug resistance integration loci described by Matzner *et al.* (36).

In conclusion, the cause for HPV integration clustering seems to be rather related to the accessibility of these fragile genomic areas than due to a selection of clones that harbor integrated HPV in regions with tumor relevant genes.

Role of Integration in HPV-Mediated Transformation. The progression of HPV-induced lesions toward cancer reflects a classical selection scenario in which certain events lead to the clonal outgrowth of single cells in a heterogeneous cell population (Fig. 1). Deregulated expression of the HPV E6 and E7 genes in epithelial stem cells leads to major chromosomal instability in the respective cells at early stages of dysplasia. This instability becomes manifest in numerical centrosome aberrations, anaphase bridges, and chromosome breaks that over time result in aneuploidy (4, 37). Hopman *et al.* (38) have analyzed DNA ploidy and HPV integration by fluorescence *in situ* hybridization in a number of clinical samples and observed a high correlation of aneuploid cells with integrated HPV genomes in high-grade dysplastic lesions. Obviously, HPV integration is facilitated by repair processes activated in these chromosomally unstable cells. The association of DNA repair and viral integration has also been described for retroviruses (39). Recently, it has been shown in a large series of clinical samples that DNA aneuploidy clearly precedes HPV integration in the progression of HPV-associated cervical precancers,[3] supporting the concept that integration occurs as a result of chromosomal repair mechanisms. Accordingly, HPV integration is most frequently observed in unstable areas of the genome that are also targeted by integration of foreign DNA molecules in other scenarios. Along with integration of HPV genomes or fragments thereof that presumably occur in parallel in multiple cell clones, selection processes seem to be initiated that finally result in preferred outgrowth of only one or few cell clones with optimized expression of the HR-HPV oncogenes (16). Finally, a malignant cell clone might emerge that accounts for the majority of the evolving tumor mass and that can also be found in local recurrences and distant metastases.[4] Therefore, the detection of HPV integration points to progressing lesions and might be applied in various

[3] P. Melsheimer. DNA aneuploidy precedes integration of HPV16 E6/E7 oncogenes in intraepithelial neoplasia and invasive squamous cell carcinoma of the cervix uteri, submitted for publication.

[4] S. Vinokurova. Clonal composition of HR-HPV induced high grade cervical dysplasia and invasive carcinomas, manuscript in preparation.

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clinical applications: it can be a valuable individual tumor and recurrence marker. Detection of specific integration sites in biopsies, *e.g.,* from lymph nodes or potential distant metastases, can be used as a tumor staging tool. Posttreatment detection of residual cells with identical integration patterns as the primary tumor indicates residual disease and might significantly influence the therapeutic decision taking.

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