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Synchronous cancers involving the ovaries and the uterine corpus are well-known events in gynecologic malignancies.<sup>1,2</sup> These tumors can be independently derived, non-metastatic tumors (dual primary tumors [DP]) or a tumor from 1 organ with metastasis to another (single primary tumor with metastasis [SPM]). In most of these synchronous endometrial and ovarian cancers, both tumors are diagnosed histologically as endometrioid adenocarcinomas, which may complicate the distinction between DP and SPM. Among 326 cases of endometrial carcinoma encountered at the University of Tokyo Hospital since 1999, 27 (8.3%) were synchronous endometrial and ovarian cancers. Of these 27 synchronous carcinomas, 9 were histopathologically diagnosed as DP and 18 as SPM.

Correct diagnosis of DP and SPM is clinically very important because the prognosis of DP is significantly better than that of SPM.<sup>3–5</sup> Using the guidelines according to the International Federation of Gynecology and Obstetrics,<sup>6</sup> DP tumors, when confined to the ovary (or ovaries) and uterine corpus alone, would represent 2 stage I cancers. Patients with DP may not require any adjuvant chemotherapy or radiotherapy, depending on the stage of each tumor. On the other hand, a primary endometrial cancer with ovarian metastases would be classified at least as stage IIIa, and a primary ovarian cancer with metastasis to the uterine corpus would be at least stage IIa. These patients with SPM require more aggressive treatment, including adjuvant chemotherapy (and/or radiotherapy). Thus far, the diagnosis of synchronous endometrioid adenocarcinomas has been mainly based on multiple pathological features, including tumor grade, extent of myometrial invasion, vascular invasion, ovarian tumor size, pattern of ovarian involvement, and presence or absence of precursor lesions (such as atypical endometrial hyperplasia and ovarian endometriosis).<sup>7–10</sup> However, similar histology can-

not be used as evidence of metastasis from one organ to another. Two tumors may have a similar histological appearance but could still represent independent tumors. Conversely, a metastatic tumor may appear to be dissimilar from the primary tumor owing to morphologic variations in either tumor, such as dedifferentiation and epithelial-mesenchymal transition. Thus, a nondefinitive diagnosis between SPM and DP might result in an inaccurate prognostic evaluation and consequently unsuitable adjuvant treatments (mostly excessive treatments) in a significant proportion of synchronous tumors.

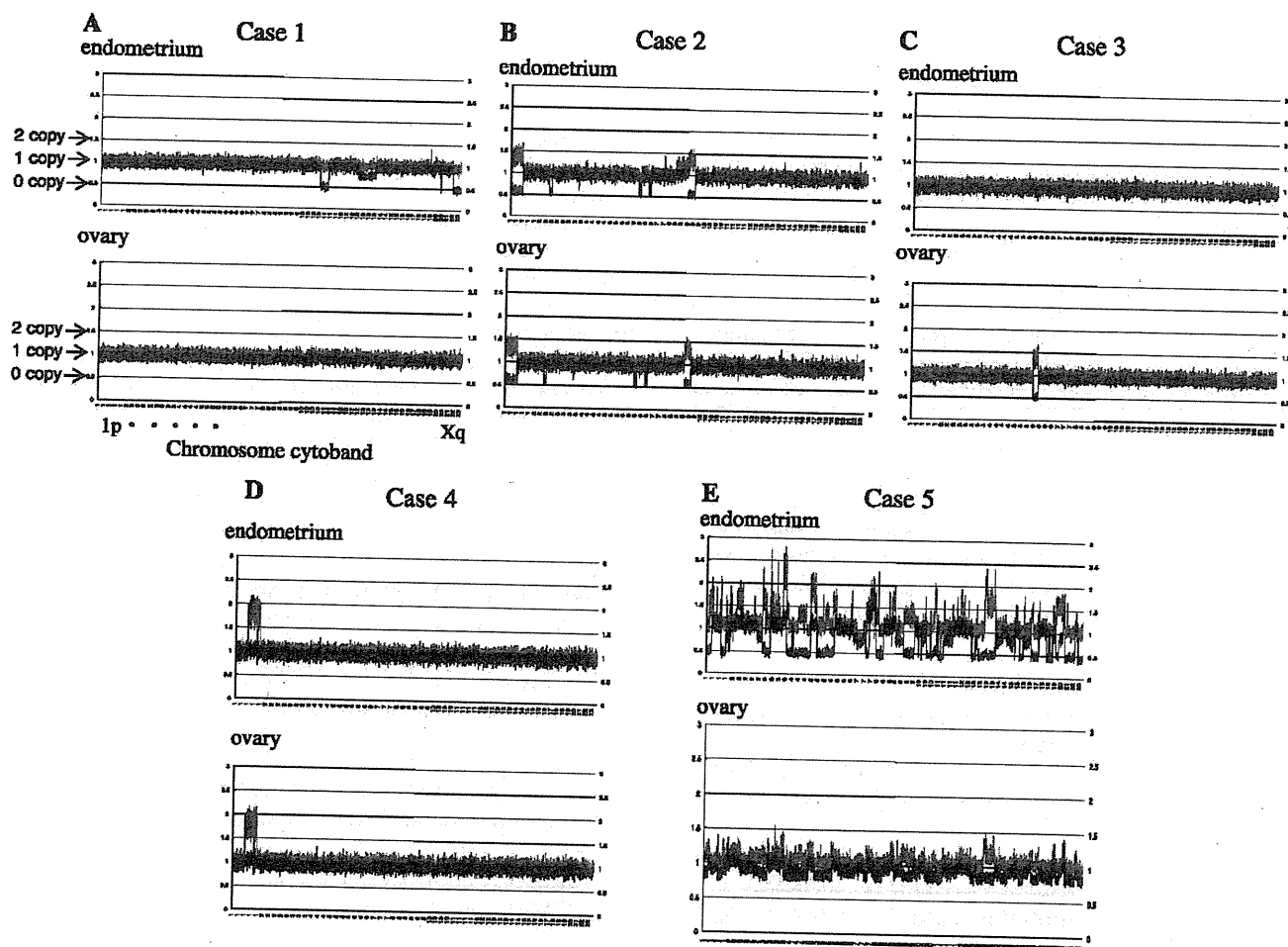
To increase the accuracy of the diagnosis, a number of attempts have been made to characterize synchronous tumors based on their molecular alterations. The approaches include loss of heterozygosity (LOH), X chromosome inactivation, microsatellite instability (MSI), and mutational analysis of *PTEN* and *CTNGB1*.<sup>11–15</sup> However, distinguishing primary cancer from metastatic cancer is still challenging because additional molecular changes in either the primary or the metastatic tumor can obscure the genetic identity. Genome-wide genotyping, such as single nucleotide polymorphism (SNP) arrays, has improved greatly and has revealed chromosomal copy number alterations (CNAs) throughout the genome in a single assay.<sup>16</sup> Allele-specific copy number information by SNP arrays has unveiled copy number-neutral (CNN) LOH (loss of 1 allele and gain of the opposite allele) in various types of tumors, including endometrial and ovarian carcinomas.<sup>17,18</sup>

In this study, we assessed whether SNP array genotyping is useful for diagnosing synchronous endometrioid adenocarcinomas of the uterine corpus and the ovary. In addition, we validated the SNP array genotyping diagnoses by analyzing the microsatellite status and genetic mutations of several cancer-related genes.

TABLE 1. Clinicopathological features and diagnosis

Case	Age, y	Menopause	Gravidity	Parity	BMI, kg/m <sup>2</sup>	Endometrial Cancer							
						Tumor Size, cm	Grade	MI	EMH	LVI	Cx	ER	PgR
1	56	54	0	0	14.3	8 × 4	2	>2/3	–	+	+	–	–
2	47	—	1	1	21.7	2 × 1, 4 × 2	1	>1/3, <2/3	–	+	+	–	–
3	52	50	0	0	19.6	3 × 1	1	<1/3	–	–	–	+	2+
4	32	—	0	0	22.9	7 × 5	1	>2/3	–	–	+	–	+
5	51	50	1	1	19.9	4 × 4	3	>1/3, <2/3	–	+	–	2+	3+

BMI, body mass index; MI, myometrial invasion; EMH, endometrial hyperplasia; Cx, cervical involvement.



**FIGURE 1.** Single nucleotide polymorphism array "karyograms" of 10 tumors from 5 synchronous endometrial and ovarian carcinomas. The signal intensity ratio between the raw signal intensity from the cancer and paired normal samples is ordered by position in the genome, beginning at 1p and ending with X. The top panel shows allele-specific copy number in the endometrial carcinoma, and the bottom panel is that in the paired ovarian carcinoma. A, B, C, D, and E correspond to cases 1, 2, 3, 4, and 5, respectively.

Tumor Size, cm	Tumor Site	Ovarian Cancer			Presumed Origin	ER	PgR	Other Sites of Involvement		Pathological Diagnosis	
		Endometriosis	LVI	Grade				Lymph Nodes	Peritoneum	Stage (Uterus)	Stage (Ovary)
6 × 5	Inside	—	—	1	Endometrium	—	2+	—	—	IIIa	—
3 × 4	Inside	—	—	1	Ovary	—	2+	—	—	IIb	Ia
15 × 11	Inside	(Adenomyosis)	—	1	Ovary	+	+	—	—	Ia	Ia
4 × 3	Inside	+	—	1	Ovary	—	2+	+	+	IIIc (pT3a N1 M0)	La
5 × 4	Inside + surface	—	+	3	Endometrium	—	—	—	+	IIIa	—

## MATERIALS AND METHODS

### Tumor Samples and Genomic DNA

In total, 10 surgical specimens were obtained from 5 patients with synchronous endometrial or ovarian endometrioid adenocarcinomas who underwent resection of their tumors at the University of Tokyo Hospital. All the patients provided informed consent for the collection and use of their samples for research, and the use of tissues for this study was approved by the appropriate institutional ethics committees. The clinicopathological features of the 5 cases are detailed in Table 1. Treatment protocols for endometrial carcinomas were described previously.<sup>18</sup> All of the patients received primary surgery, followed by 6 cycles of platinum-based chemotherapy. The fresh frozen tumors were embedded in optimal cutting temperature compound and were cut into 4- $\mu$ m tissue sections, which were stained with hematoxylin and eosin. Sections with high content and purity of tumor epithelium were used for DNA extraction. Genomic DNA was isolated from the tumor sections or lymphocyte pellets using a QIAamp DNA Easy Kit (Qiagen, Valencia, CA) according to the manufacturer's specifications.

### Immunohistochemistry

Immunohistochemistry for estrogen receptor (ER) and progesterone receptor (PgR) was performed, and the intensity of staining in tumor cells was scored independently (0–3) by 2 investigators as described previously (Y.T. and D.M.).<sup>19</sup>

### SNP Array and Genome Imbalance Map

Single nucleotide polymorphism array was performed in the 10 synchronous carcinomas from the 5 patients with paired control DNA. Experimental procedures for GeneChip were performed according to the GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA), using a Human Mapping 250K Nsp Array (Affymetrix). The Genome Imbalance Map algorithm was applied to the raw data of endometrial (or ovarian) cancer and peripheral blood obtained from SNP arrays as described previously.<sup>18,20</sup> The purity of tumor epithelium at 50% was previously confirmed to be sufficient for copy number evaluation in SNP arrays.<sup>20</sup> One ovarian cancer specimen (case 5) contained more normal DNA contamination than the others did, and cut-off ratios of greater than 1.15 for gain and less than 0.85 for loss were used in each region. We classified tumors with 5 or more loci of copy alterations as chromosomal instability (CIN)-extensive, those with 1 to 4 loci as CIN-intermediate, and those without any copy alterations as CIN-negative.

### Polymerase Chain Reaction and Sequencing

Mutational analysis of *PIK3CA*, *PTEN*, *K-Ras*, and *CTNNB1* was performed. Mutations for *PTEN* (exons 1–8), *K-Ras* (exons 1 and 2), and *PIK3CA* (exons 9 and 20) were analyzed as previously described.<sup>21–23</sup> The primer sequences of exon 3 for the *CTNNB1* gene were as follows: forward, 5'-ATTTGATGGAGTTGGACATGGC-3' and reverse, 5'-CCAGCTACTTGTCTTGTGAG-3'. The polymerase chain reaction products were sequenced using the BigDye

**TABLE 2.** Genetic mutation status in *PIK3CA*, *PTEN*, *K-Ras*, and *CTNNB1* and loci of chromosomal CNAs in dual site carcinomas of the uterus and ovary

Case		Genetic Mutations			
		<i>PIK3CA</i>	<i>PTEN</i>	<i>K-Ras</i>	<i>CTNNB1</i>
1	EM	<b>E365K</b>	<b>D24Y, R142W</b>	WT	WT
	OV	<b>E81K</b>	<b>R15K, F81C</b>	WT	WT
2	EM	WT	WT	WT	<b>S36C</b>
	OV	WT	WT	WT	<b>S36C</b>
3	EM	<b>N345D, Y1021C</b>	WT	WT	WT
	OV	<b>N345D, Y1021C</b>	WT	WT	WT
4	EM	<b>H1047R</b>	WT	WT	<b>S32C</b>
	OV	<b>H1047R</b>	WT	WT	<b>S32C</b>
5	EM	<b>M10431</b>	WT	WT	WT
		<b>M10431</b>	WT	WT	WT
	OV	<b>M10431</b>	WT	WT	WT

EM, endometrial cancer; OV, ovarian cancer; WT, wild type. Bold values signify the existence of genetic mutations in the tumors.

Terminator (Applied Biosystems, Foster City, CA) method on an autosequencer.

### MSI Analysis

Microsatellite instability status was analyzed using 5 fluorescence-labeled microsatellite markers (BAT25, BAT26, D2S123, D5S346, and D17S250) as described previously.<sup>18</sup> Microsatellite instability status was determined as follows: tumors with 2 or more positive MSI markers as MSI-high, 1 positive MSI marker as MSI-low, and no positive MSI markers as microsatellite stable.

## RESULTS

### Histopathological Diagnoses in Synchronous Ovarian and Endometrial Cancers

Five patients with synchronous endometrial and ovarian cancers were diagnosed based on multiple pathological features and previously proposed criteria (Table 1).<sup>8,10</sup> Two patients (cases 2 and 3) were pathologically diagnosed as SPM and 3 as DP. All 3 DP cases were diagnosed as stage I ovarian cancer with stage I to III endometrial cancer (Table 1). The 2 endometrial SPM cases were diagnosed as stage IIIa or IIIc (pT3a by ovarian metastases). Tumor grade, lymphovascular invasion (LVI), and expression of ER and PgR were concordant between the endometrial and the ovarian carcinomas in 4, 3, 4, and 2 patients, respectively (Table 1). These similarities and variations reflect the diagnostic complexity of the synchronous tumors.

### SNP Array Genotyping in Synchronous Endometrial and Ovarian Adenocarcinomas

The genome-wide copy number imbalances were compared between the endometrial and the corresponding ovarian carcinomas in these patients. All 5 patients showed 1 or more copy number imbalances in 1 or in both synchronous endometrial and ovarian carcinomas (Fig. 1A–E). Of the 5 paired tumors, 3 (cases 2, 4, and 5) exhibited concordant patterns of copy number imbalances (Fig. 1B, D, and E). The regions of each imbalance in the 3 paired tumors were identical between the synchronous tumors (Table 2 and Supplemental Digital Content, Table 1, <http://links.lww.com/IGC/A90>). One case (case 1) exhibited CNAs at 4 loci specifically in the endometrial carcinoma, whereas the ovarian carcinoma contained no CNAs (Fig. 1A). Another case (case 3) demonstrated 1 CNN LOH in the ovarian carcinoma but not in the endometrial carcinoma (Fig. 1C). These data suggest that SNP array is a useful method to compare copy number imbalances throughout the genome between synchronous endometrial and ovarian adenocarcinomas.

### Status of MSI and Genetic Mutations in Synchronous Endometrioid Adenocarcinomas

Microsatellite instability was analyzed in the synchronous endometrial and ovarian carcinomas. Microsatellite instability-high status was detected in 1 (case 3) of the 5 patients. All 5 microsatellite markers were positive in both the endometrial and ovarian carcinomas in this patient (Supplemental Digital Content, Table 2, <http://links.lww.com/IGC/A91>). No

Loci of Chromosomal CNAs

Loci of Gain	Loci of Loss	CNN LOH	CIN	Discordant Loci
None	11p, 13q, 20q, X	None	Intermediate	Loss at 11p, 13q, 20q, X
None	None	None	Negative	None
None	2q, 6q13, 6q25	1p, 9p	Extensive	None
None	2q, 6q13, 6q25	1p, 9p	Extensive	None
None	None	None	Negative	CNN LOH at 6p
None	None	6p	Intermediate	None
1q	None	None	Intermediate	None
1q	None	None	Intermediate	None
1p, 1q, 2p, 2q, 3p, 3q, 5p, 5q, 6q, 7q, 8q, 10q, 11p, 11q, 12p, 12q, 15q, 16q,	1p, 1q, 3p, 4p, 4q, 5p, 5q, 7q, 8p, 10p, 10q, 11p, 12q, 13, 16q,	1p, 1q, 3p, 4p, 4q, 5q, 6p, 7q, 8p, 7q, 8p, 9q, 10q, 13q, 17p, 17q,	Extensive	None
1p, 1q, 2p, 2q, 3p, 3q, 5p, 5q, 6q, 7q, 8q, 10q, 11p, 11q, 12p, 12q, 15q, 16q,	1p, 1q, 3p, 4p, 4q, 5p, 5q, 7q, 8p, 10p, 10q, 11p, 12q, 13, 16q,	5q, 6p, 7q, 8p, 9q, 10q, 13q, 17p, 17q, 20p, 21q	Extensive	None

positive microsatellite markers were detected and diagnosed as microsatellite stable in the other 4 patients.

Among the 5 synchronous endometrial carcinomas, we detected *PIK3CA* mutations in 4 (80%), *PTEN* mutations in 1 (20%), and *CTNNB1* ( $\beta$ -catenin) mutations in 1 patient (20%). No *K-Ras* mutations were detected in these samples. Both endometrial and ovarian carcinomas in case 1 possessed coexistent mutations in *PIK3CA* and *PTEN*; however, no mutation sites were overlapped between the 2 tumors (Table 2). In contrast, the other 4 synchronous tumors harbored identical mutations in the endometrial and ovarian carcinomas (Table 2). We confirmed that all these mutations were somatic by analyzing the corresponding normal DNA (Supplemental Digital Content, Figure 1 and legend, <http://links.lww.com/IGC/A87>, <http://links.lww.com/IGC/A88>).

### Discrepancy Between Pathological and Genetic Diagnoses

The histopathological diagnosis and all the genetic diagnostic data are summarized in Table 3. Single nucleotide polymorphism array genotyping and genetic mutations were identical in 3 (cases 2, 4, and 5) of the 5 cases, indicating that these synchronous tumors were SPM. In case 3, the 2 point mutations in the *PIK3CA* gene and alterations of microsatellite markers were the same in the endometrial and the ovarian carcinomas. These data suggest that the case was more likely to be SPM and that the CNN LOH detected only in the ovarian carcinoma might have occurred after metastasis to the ovary. Case 1 was genetically diagnosed as DP.

### DISCUSSION

In this study, we applied a genome-wide genotyping approach by SNP typing arrays to diagnose whether 2 tumors in the endometrium and ovary in the same patient represent DP or SPM. Among gynecologic synchronous tumors, endometrioid adenocarcinomas of the endometrium and ovary are clinically important for the following reasons: (1) this combination is the most frequent among gynecologic synchronous malignancies, (2) histopathological diagnosis is very difficult owing to the morphologic similarities between

the tumors, and (3) the prognosis is much better in DP (with early stage of each tumor) than in SPM (with more advanced stage of the single primary tumor).

Genome-wide genotyping technologies have now become feasible for practical use in cancer therapy and diagnosis.<sup>24</sup> Microarray gene expression profiling (GeneChip; Affymetrix) is used for genome-wide analyses, but the results can be significantly affected by normal cell contamination. In contrast, purity of tumor epithelium of 50% is sufficient for copy number evaluation in SNP arrays.<sup>20</sup> In this study, we demonstrated that allele-specific SNP array genotyping is a useful diagnostic methodology in synchronous endometrial and ovarian carcinomas. The information about CNAs that can be obtained throughout the genome includes (1) the type of alterations (gain, loss, or CNN LOH), (2) the locus and the length (minimal regions) of each alteration, and (3) the degree of each CNA. Thus, the diagnosis would be more definitive with a larger number of CNAs, as seen in cases 2 and 5 in this study. Concordant "karyograms" in the SPM cases suggest that CNAs might occur mainly before metastasis and that subsequent alterations after the metastasis might not be frequent in SPM. However, the limitation of diagnosis by SNP arrays should be considered because secondary changes cannot be excluded, as observed in case 3. Using SNP typing arrays, we previously reported that CIN (CIN-extensive, CNAs with 5 or more loci) is an independent poor prognostic factor in endometrial cancer.<sup>18</sup> Taken together with the present results, this suggests that SNP array genotyping might be useful to predict poor prognostic patients with synchronous endometrial and ovarian carcinomas via the evaluation of clonality and CIN.

Assessment of the status of genetic mutations and microsatellite markers was helpful for the validation of the diagnosis by SNP arrays. We selected 4 genes (*K-Ras*, *PTEN*, *PIK3CA*, and *CTNNB1*) in this study, which are commonly mutated in endometrial carcinomas.<sup>23,25</sup> Among the 5 sets of synchronous endometrial and ovarian carcinomas, discordant mutational patterns were only detected in case 1, which was diagnosed as DP by the SNP arrays. The mutational data should also be applied with careful consideration because identical "hot spot" mutations in these genes might occur independently in each DP tumor.

TABLE 3. Pathological diagnosis and genetic diagnosis

Case	Pathological Diagnosis	Chromosomal Instability	Genetic Mutations (Mutated Genes)	MSI (Ut/Ov)	Genetic Diagnosis	Tumor Stage
1	SPM	Different; Ut; intermediate (4)/Ov; negative	Different (different mutations both in <i>PTEN</i> and <i>PIK3CA</i> )	Low/low	DP	EM, IIb; OV, Ic
2	DP	Identical; extensive (5)	Identical ( <i>CTNNB1</i> )	Low/low	SPM	EM, IIIa
3	DP	Different; Ut; negative/Ov; intermediate (1)	Identical ( <i>PIK3CA</i> )	High/high	SPM	EM, IIIa
4	DP	Identical; intermediate (1)	Identical ( <i>PIK3CA</i> , <i>CTNNB1</i> )	Low/low	SPM	EM, IIIc
5	SPM	Identical; extensive (>20)	Identical ( <i>PIK3CA</i> )	Low/low	SPM	EM, IIIa

Values in parentheses indicate number of loci of copy number imbalances. Ut, endometrial cancer; Ov, ovarian cancer.

In conclusion, our data revealed that each copy number imbalance was well preserved between the endometrial and the paired ovarian tumors in SPM, suggesting that genome-wide SNP typing arrays might be a useful method to diagnose synchronous endometrial and ovarian carcinomas comprehensively.

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# Productive lifecycle of human papillomaviruses that depends upon squamous epithelial differentiation

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Human papillomaviruses (HPVs) target the stratified epidermis, and can cause diseases ranging from benign condylomas to malignant tumors. Infections of HPVs in the genital tract are among the most common sexually transmitted diseases, and a major risk factor for cervical cancer. The virus targets epithelial cells in the basal layer of the epithelium, while progeny virions egress from terminally differentiated cells in the cornified layer, the surface layer of the epithelium. In infected basal cells, the virus maintains its genomic DNA at low-copy numbers, at which the viral productive lifecycle cannot proceed. Progression of the productive lifecycle requires differentiation of the host cell, indicating that there is tight crosstalk between viral replication and host differentiation programs. In this review, we discuss the regulation of the HPV lifecycle controlled by the differentiation program of the host cells.

**Keywords:** HPV, differentiation, epithelial cell, keratinocyte

## INTRODUCTION

Human papillomavirus (HPV) infections of the anogenital organs are a very common “sexually transmitted disease (STD).” Although the incidence of cancer progression is low, a HPV infection is frequently detected in anogenital cancers. As for cervical cancer, HPV DNA is detected in more than 90% of cases. Approx. 5.5 million new cases of HPV infection are reported and there are c.a. 450,000 diagnoses of cervical cancer per year worldwide, leading to approximately 200,000 deaths each year, which ranks second among cancers in women (Parkin and Bray, 2006). HPV infections have also been associated with the head and neck squamous cell carcinomas (HNSCCs).

Human papillomavirus is categorized as a small virus containing DNA. More than 120 types of HPV have been identified and one-third of them target mucosal membranes, the remainder target the cutaneous membranes. Mucosa-tropic HPVs can be classified into two types based on their association with malignant carcinomas: a high-risk type (such as HPV type 16, 18, 31) and a low-risk type (such as HPV6 and 11; Howley, 1996). Prophylactic vaccines for HPV16 and 18, Cervarix (GlaxoSmithKline), and for HPV6, 11, 16, and 18, Gardasil (Merck & Co.), have been developed recently and effectively prevent primary infections. They, however, cannot be used as therapeutic vaccines, indicating the importance of a Pap smear and the development of effective treatment strategies (Carter et al., 2011). In order to inhibit HPV-induced cancer, an understanding of the molecular basis of the infection and the characteristics of the infected lesions is important.

## GENOME ORGANIZATION OF HPV AND FUNCTIONS OF VIRAL PROTEINS

Human papillomaviruses have a common gene organization (Figure 1): an early region encoding non-structural genes, the late

region for structural genes, and a regulatory region (long control region: LCR).

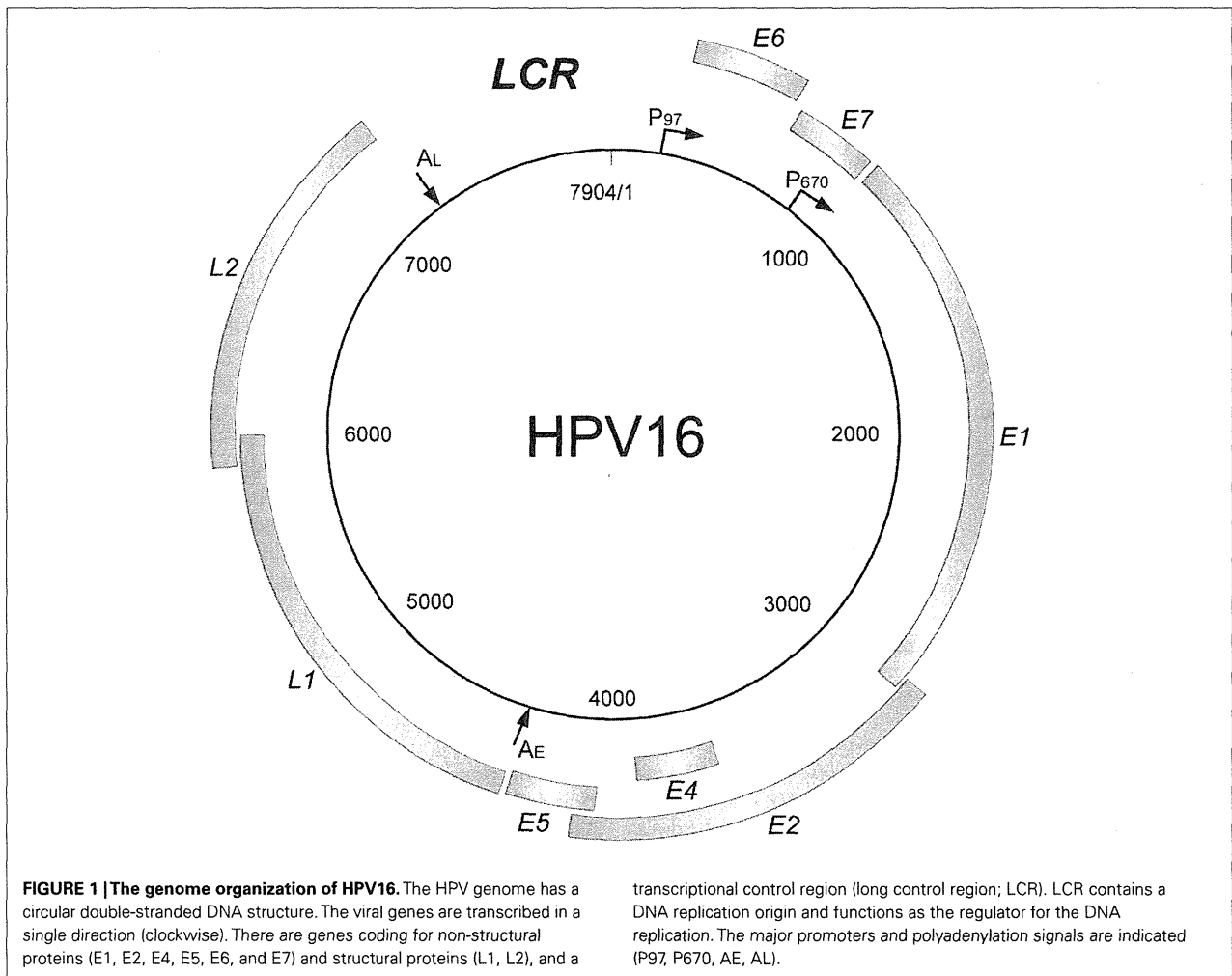
The functions of each viral protein are summarized in Table 1. E1 and E2 are cooperatively involved in the initiation of viral DNA replication. E2 also functions as a transcriptional transactivator. E6 and E7 modulate the cell cycle control and contribute to viral genome maintenance (Frattini et al., 1996; Stubenrauch et al., 1998; Thomas et al., 1999). They also contribute to cancer development (Münger et al., 2004). Though E4 and E5 are speculated to modulate the productive phase of the HPV lifecycle, their biological roles remain unclear (Fehrman et al., 2003; Genter et al., 2003; Nakahara et al., 2005; Wilson et al., 2005, 2007; Fang et al., 2006). Both L1 and L2 are capsid proteins.

## HPV LIFECYCLE

The target of a HPV infection is the stratified epithelium. In the normal stratified epithelium, the cell attached to the basal membrane (basal cell) is the only cell that has the potential to proliferate. The basal cell divides into a new basal cell and a daughter cell that is detached from the basal membrane, and the daughter cell launches its differentiation process. The daughter cells exit from the cell cycle and change their gene expression pattern, proceeding to terminal differentiation, then peel off from the epithelium (Jones et al., 2007). The lifecycle of HPV is tightly regulated by the differentiation program of the host cells (Figure 2). In this section, the differentiation-dependent lifecycle of HPV is briefly summarized.

## ENTRY OF HPV INTO THE BASAL CELLS OF STRATIFIED EPIDERMIS

Human papillomavirus virions invade through damaged areas of the epithelium and infect the basal cells. Although the receptor for the HPV infection has not been fully characterized, the following



model has been postulated; virions initially attach to the heparan sulfate proteoglycan (HSPG) on the basal membrane, and transfer to the receptor expressed on the keratinocytes moving on the basal membrane in the wound-healing process, then enter the cells (Kines et al., 2009).

#### LOW-LEVEL EXPRESSION OF VIRAL GENES AND GENOME MAINTENANCE IN THE BASAL LAYER

Following viral entry and uncoating, HPV genomic DNA is transported into the nucleus and maintained at a low-copy number in the basal cells (50 ~ 100 copies per cell; in the basal layer, **Figure 2**; Moody and Laimins, 2010). Genome maintenance as episomal status is essential for the establishment of the early phase of the viral lifecycle (McBride et al., 2006).

#### PRODUCTIVE REPLICATION OF HPV IN THE DIFFERENTIATED CELLS

After leaving the basal membrane, the infected cells initiate the differentiation program. Because HPV does not encode DNA polymerase activity for viral genome replication, the host DNA replication machinery is required. However, the DNA replication activity is suppressed in the differentiated cells that exit from the

cell division cycle. To ensure that the viral genome is replicated, HPV needs to reactivate cell division among the differentiation-initiated cells. E6 and E7 inactivate p53 and retinoblastoma protein (pRb), respectively, which enables the cells to maintain their DNA replication potential (Münger et al., 2004).

In the upper layers of the stratified epithelium (in the spinous layer, **Figure 2**), the expression of viral genes that are required for viral genome replication is markedly accelerated (Hummel et al., 1992; Ozbun and Meyers, 1997), inducing viral genome amplification to thousands of copies per cell (Bedell et al., 1991). Following the genome amplification, in the terminally differentiated cells, the synthesis of capsid proteins is triggered. The capsid proteins assemble into virions that encapsidate viral genomic DNA. The progenitor virions are released externally with peeled keratinocytes.

#### DIFFERENTIATION-DEPENDENT CONTROL OF HPV LIFECYCLE

The differentiation-dependent lifecycle of HPV is controlled of multiple levels, such as transcription, post-transcriptional processing, translation, and DNA replication. In the following sections, each regulatory mechanism is summarized.



Table 1 |

Function in viral lifecycle	Activities	Target factor
<b>E1</b>		
Replication of viral genome	DNA-binding activity, helicase activity, ATPase	RPA, topoisomerase, polymerase alpha-primase
<b>E2</b>		
Transcription of viral genes		
Replication of viral genome	Transactivation/transrepression, DNA-binding	Brd4, ChlR1
Maintenance of viral genome	activity, DNA segregation in mitotic cell	
<b>E6</b>		
Reactivation of cellular replication mechanisms		
Proliferation, immortalization, inhibition of apoptosis	Interaction with various cellular proteins	<b>p53</b> , ADA3, p300/CBP, E6AP, SP1, c-Myc, NFX1-91, TERT, FAK, FADD, Caspase 8, BAX, BAK, IRF3, PDZ domain proteins
Maintenance of viral genome		
<b>E7</b>		
Reactivation of cellular replication mechanisms		
Proliferation, genomic instability, inhibition of apoptosis	Interaction with various cellular proteins	<b>RB</b> , p107, p130, HDAC, E2F6, p21, p27, CDK/cyclin, ATM, ATR, gamma-tubulin
Maintenance of viral genome		
<b>E4</b>		
?		
	Destruction of keratin network, induction of G <sub>2</sub> M arrest of cell cycle	Cytokeratin 8/18
<b>E5</b>		
?		
Proliferation? Inhibition of apoptosis?	Affection of cellular signaling pathway	EGFR, PDGFR, V-ATPase, MHC1, TRAIL receptor, FAS receptor
<b>L1</b>		
Major capsid protein		
<b>L2</b>		
Minor capsid protein		

### TRANSCRIPTIONAL REGULATION OF VIRAL GENES

Human papillomavirus has two major promoters, the early promoter and the late promoter. In HPV16, P97, and P670 have been identified as the early and late promoters, respectively (Figure 1). Transcriptional activity is mainly controlled by the LCR. A transcriptional enhancer is located within the LCR, with which various cellular transcription factors can associate (Figure 3).

The binding sites for the viral transcriptional regulator, E2, are found in HPV16 LCR. Viral gene expression is regulated by the occupancy status of the E2-binding sites (E2BSs; Figure 3), which is partly defined by the E2 expression level controlled by cellular differentiation status (Steger and Corbach, 1997; Hadaschik et al., 2003).

E2 functions in viral genome segregation by tethering the viral DNA to the mitotic chromatin, in which a cellular protein, bromodomain-containing protein 4 (Brd4), has been reported to be involved (McPhillips et al., 2006). Interaction between E2 and Brd4 is also required for the E2-mediated transcriptional activation and repression (McPhillips et al., 2006; Wu et al., 2012).

A ubiquitous transcription factor, Sp1 is a well-known regulator for HPV gene expression. The Sp1-binding site partially overlaps with one of the E2BSs (E2BS#2), and a TATA box element is located close to the promoter-proximal E2BS (E2BS#1; Figure 3). The binding of E2 to those E2BSs, therefore, interferes

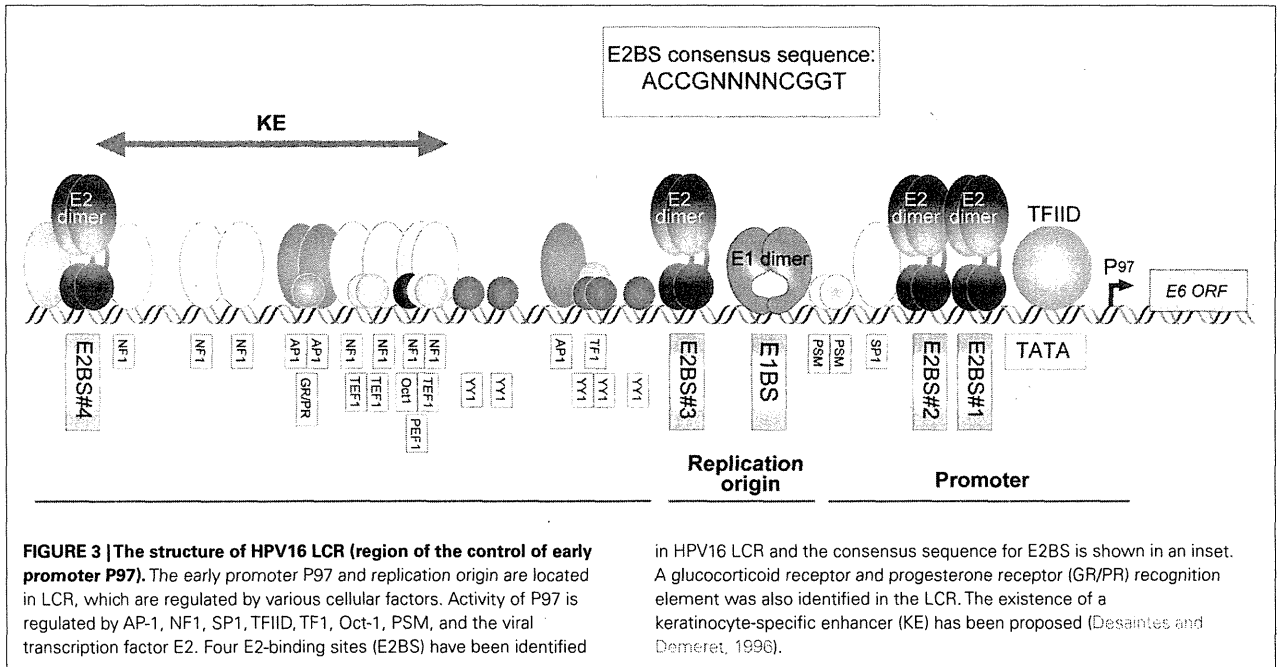
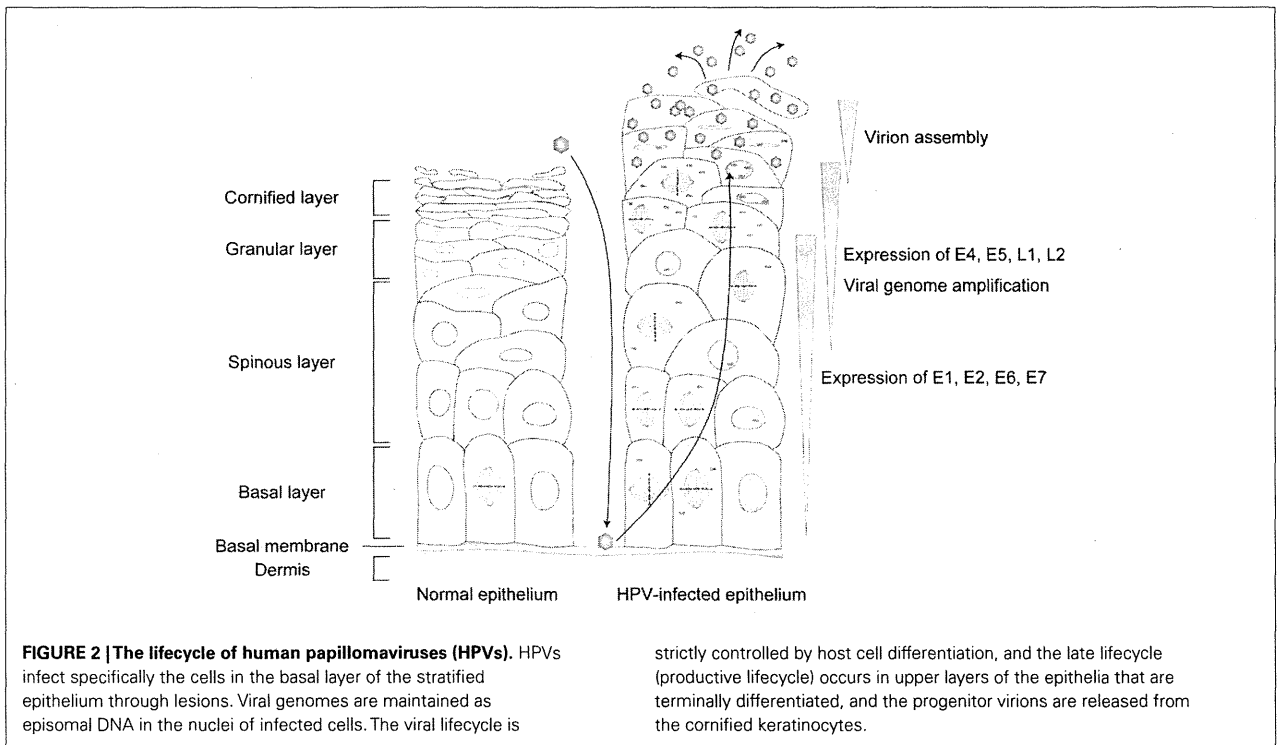
with the assembly of the transcriptional initiation complex, resulting in a suppression of E6/E7 expression that is governed by the early promoter activity (Tan et al., 1992). It was also reported that Sp1 altered the chromatin structure of HPV16 LCR, offering the accessibility of transcription factors (Stümel and Bernard, 1999).

### TRANSCRIPTIONAL CONTROL IN THE UNDIFFERENTIATED CELLS

Transcripts of viral early genes are expressed in the infected basal cells, which is essential for the viral DNA replication (Dürst et al., 1992). It was reported that a unique promoter, P14, was utilized for E1 expression and the E2BSs were considered as necessary for the P14 activity (Lace et al., 2008). The transcript initiated from P14 is a poly-cistronic mRNA containing E6, E7, and E1, in which the shunting in ribosomal scanning process enables the translation of E1 (Remm et al., 1999). The regulatory mechanism for E2 expression has not been clarified. The early promoter is used for E6 and E7 expression, in which several transcription factors, including AP-1, glucocorticoid receptor, NF1, Oct-1, Sp1, YY-1, and CDP, are involved (Figure 3; Desaintes and Demeret, 1996).

### TRANSCRIPTION IN THE DIFFERENTIATED CELLS

The early promoter is activated in association with the differentiation process, increasing the E1/E2 expression (Hummel et al., 1992; Ozburn and Meyers, 1997). Although levels of E6 and E7 also



increase with the early promoter's activation, the E2 overexpressed in the upper layer is thought to suppress their transcription via the mechanism mentioned above. E6 and E7 are important in maintaining infected cells in an undifferentiated state, but terminal differentiation is required for the productive replication of HPV.

The inhibition of E6/E7 expression by E2 might promote cellular differentiation, and the cells undergo terminal differentiation, which is suitable for the viral productive lifecycle.

AP-1, a heterodimer composed of Fos and Jun, is considered to be involved in the differentiation-dependent transcriptional

control in keratinocytes; there are reports that the expression profiles of Fos and Jun family members were modified, and that the interaction between AP-1 and KRF-1, a keratinocyte-specific transcription factor (Mack and Laimins, 1991), was strengthened in the differentiation process (Desaintes and Demeret, 1996; Thierry, 2009). Several transcriptional factors were reported to be involved in the differentiation-dependent control of LCR function; EPOC-1/Skn-1a, C/EBP- $\alpha$ , - $\beta$ , c-Myb, NF1, NFATx, Pax5, and WT1 (Desaintes and Demeret, 1996; Thierry, 2009).

The late promoter is specifically activated in the differentiated layers of epithelium. The late promoter activity is suppressed by CDP (CCAAT displacement protein) and YY-1, whose binding potential was reported to be decreased in differentiated keratinocytes (Ai et al., 1999, 2000). There was also a report that the expression ratio of a transcription factor, Sp1 and its antagonist, Sp3, was altered through the differentiation, which activated the late promoter activity (Apt et al., 1996). The binding of hSkn-1a and C/EBP $\alpha$  to the proximal region of the late promoter contributes to the control of the late promoter activity (Kukimoto and Kanda, 2001; Wooldridge and Laimins, 2008). The involvement of E7 in the regulation of the late promoter activity was also described (Bodily and Laimins, 2011; Bodily et al., 2011). It still remains necessary to clarify the regulatory mechanism for the late promoter in the differentiation of epithelial cells.

**METHYLATION OF THE HPV GENOME DURING THE CELL DIFFERENTIATION PROCESS**

HPV gene expression is controlled by the methylation of HPV genomic DNA. As E2BSs contain CpG dinucleotides (see inset in Figure 3), they can be modified by DNA methylation in the host

cell. E2BSs are reported to be highly methylated in undifferentiated cells, inhibiting E2-binding, and demethylation at the E2BSs occurs in association with the cell differentiation (Kim et al., 2003; Vinokurova and von Knebel Doeberitz, 2011).

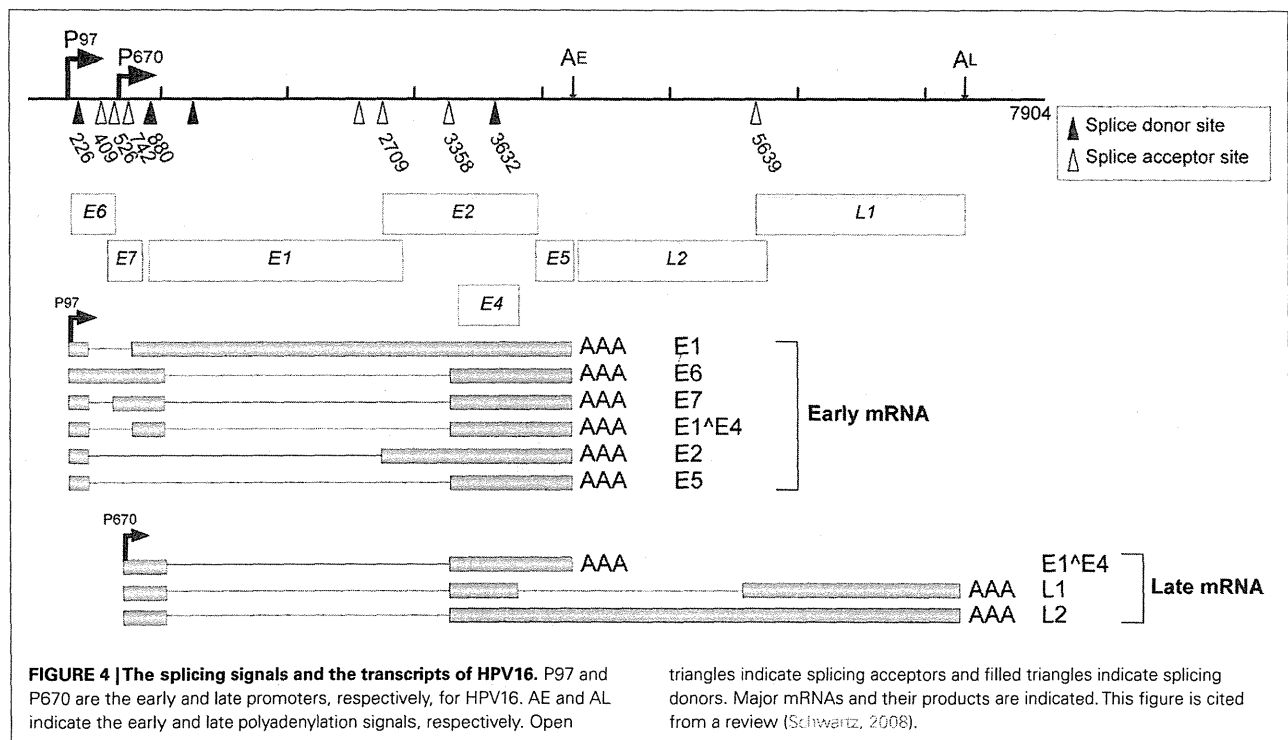
**RNA PROCESSING**

For conversion of the gene expression profile from the early to late phase of viral replication, RNA processing is considered critical. The primary transcript of HPV encodes multiple viral genes, and precise RNA processing is essential to produce the mRNA for each viral gene at an appropriate stage of cell differentiation (Schwartz, 2008).

In the early phase of the viral lifecycle, the primary transcription initiated by the early promoter is terminated at the early poly(A) signal, AE (Figure 1), and the transcript is processed by using the early splicing signals, which produces the mRNAs encoding the viral early genes. In the differentiated cells, the transcripts for the late genes are expressed from the late promoter and utilize a late poly(A) signal, AL (Figure 1), and late splicing signals. The early and late splicing signals compete for the splicing factors, so their usages are generally exclusive.

Multiple splicing signals are found in the HPV genome, which are utilized for the expressions of various viral genes (Figure 4). These splicing signals can be categorized into three groups; early phase-specific signals (DS226, SA409, SA526, SA742 in HPV16), late phase-specific signals (SD 3632 and SA5639), and non-specific signals (SD880, SA2709, SA3358; Schwartz, 2008).

Early splicing events have three major roles; regulation of the expression ratio of early genes, production of splicing variants of viral genes, and suppression of late gene expressions. The early



**FIGURE 4 | The splicing signals and the transcripts of HPV16.** P97 and P670 are the early and late promoters, respectively, for HPV16. AE and AL indicate the early and late polyadenylation signals, respectively. Open

triangles indicate splicing acceptors and filled triangles indicate splicing donors. Major mRNAs and their products are indicated. This figure is cited from a review (Schwartz, 2008).

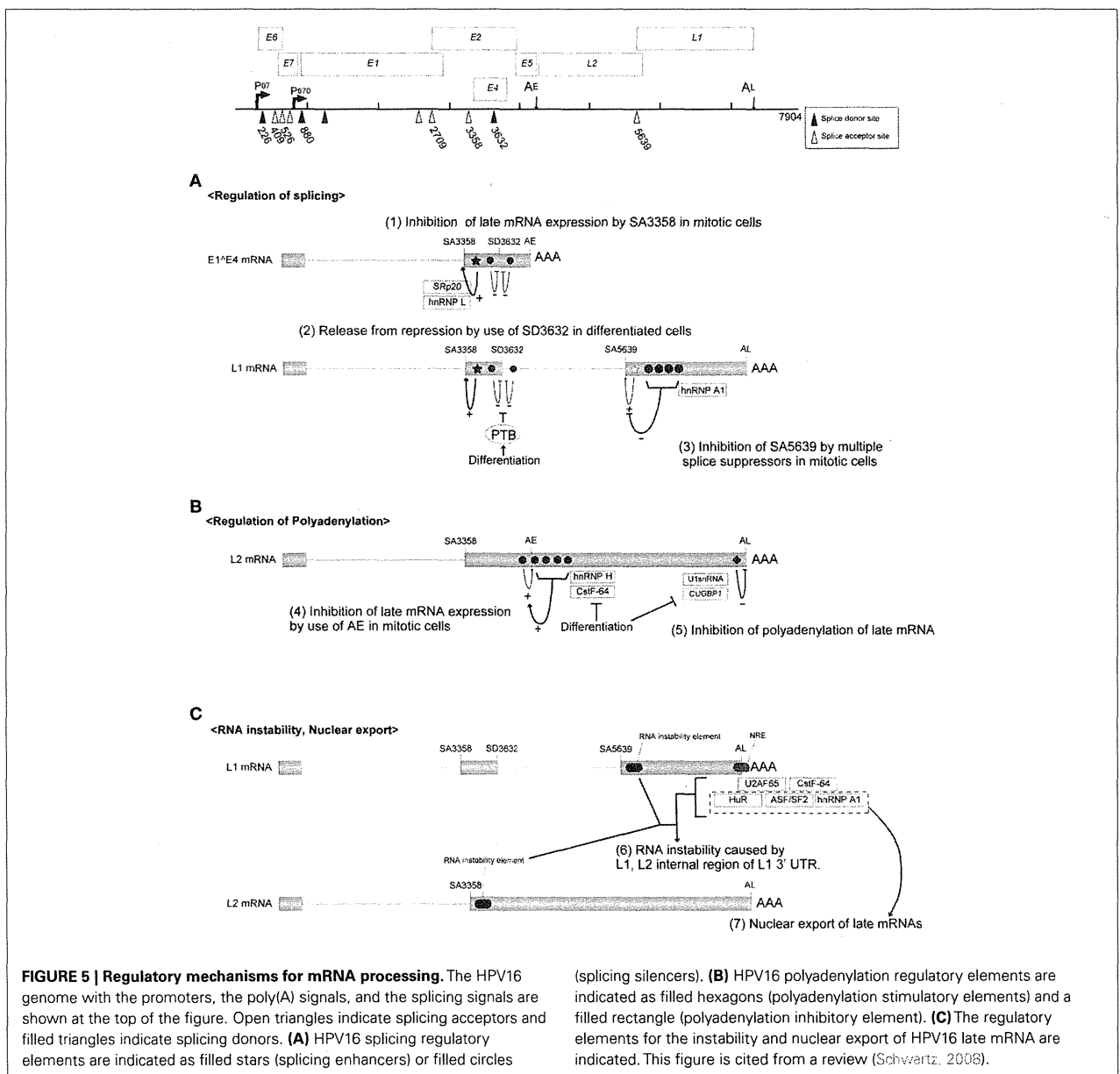
splicing sites of HPV16, SD226, SA409, and SA526, are located in the E6 ORF, which enhances the translation efficiency of E7. Because the initiation codon for E6 is leaky and there are several splicing signals in the E6 ORF, various variants of E6 can be expressed. Those variants were reported to counteract the full-length E6, which might be important for the fine-tuning of E6 activities.

SA3358 is utilized in both the early and late phases of viral replication to produce HPV16 E1<sup>+</sup>E4 mRNA. A strong splicing enhancer was identified downstream of SA3358, and it accelerated the polyadenylation at AE and suppressed the late gene expression in the early phase (Figure 5A). In undifferentiated cells, SA3358 competed with the late splicing signals for the splicing factors

(SRp20, hnRNPL etc.), which might contribute to the suppression of late gene expression (Rush et al., 2005; Jia et al., 2009).

The late mRNAs are transcribed from the late promoter, and a major splicing event occurs between SD880 and SA3358 in HPV16. For the production of L1 mRNA, additional splicing between SD3632 and SA5639 is required. The mRNAs for L1 and L2 are poly-cistronic, and the mechanism for bypassing the upstream ATG remains to be explained.

SD3632 in HPV16 is used exclusively for late mRNA production. SD3362 is located close to SA3358 and AE, and the usage of SD3362 was suppressed by a splicing suppressor in dividing cells. It was indicated that the polypyrimidine tract-binding protein (PTB) interfered with the splicing suppressor in differentiated



cells, which potentiated late gene expression (Figure 5A; Somberg et al., 2008).

A late phase-specific SA5639 in HPV16 was reported to be regulated by the cis-acting elements found in the L1 coding region (Zhao et al., 2004). A splicing enhancer was identified in the 3' region of SA5639. Although the enhancer was activated in dividing cells, its function was hindered by multiple splicing suppressors located in the L1 coding region. It was reported that hnRNP A1 is associated with those multiple splicing suppressors (Figure 5A; Zhao et al., 2007).

To produce the late mRNAs, it is essential to bypass the polyadenylation at AE. Enhanced utilization of AE could, therefore, inhibit the late gene expression. The 5' 400 nt of the HPV16 L2 ORF was reported to enhance the polyadenylation at AE, where multiple-G-motifs are located (Oberg et al., 2005). The hnRNP H interacted with the G-motifs and accelerated the polyadenylation at AE, and the expression of hnRNP H decreased as the cell differentiation progressed. CstF-64 was also reported to bind the L2 coding region of HPV31 and enhance the polyadenylation at AE, and the expression of CstF-64 diminished during the cell differentiation process (Figure 5B; Terhune et al., 2001).

Within the 3'UTR of the late mRNA (late UTR), a motif highly homologous to the U1snRNA was identified. It was reported that U1-70K, a component of U1snRNP, bound to the U1snRNA motif of BPV1 and interfered with the polyadenylation (Furth et al., 1994). Although U1-70K binding was not detected with HPV16, CUG binding protein 1 (CUGBP1) was reported to interact with the late UTR element of HPV16 and inhibit the polyadenylation process (Figure 5B; Goraczniak and Gunderson, 2008).

#### CONTROL OF LATE mRNA STABILITY

There are RNA instability elements within the L1 and L2 coding mRNAs of HPV16, which function in undifferentiated cells (Mori et al., 2006), although the mechanism for RNA destabilization remains to be clarified (Sokolowski et al., 1998; Collier et al., 2002). There is a GU-rich negative regulatory element (NRE) in the late UTR of HPV16, which is a RNA instability element (Kennedy et al., 1991). Various factors, ASF/SF2, CstF064, U2AF65, hnRNPA1, and HuR, are reported to regulate the differentiation-dependent events of HPV replication through binding to the NRE (Figure 5C; Dietrich-Goetz et al., 1997; Koffa et al., 2000; Cheunim et al., 2008).

A highly U-rich region was located in the early UTR of HPV16 and reduced mRNA stability; a U-rich region is a signature feature of unstable mRNA (Jeon and Lambert, 1995; Barreau et al., 2005).

#### NUCLEAR EXPORT OF LATE mRNAs

The L1 mRNA of HPV16 was retained in the nucleus in undifferentiated W12 epithelial cells (Koffa et al., 2000), suggesting that the nuclear export of late mRNAs was inhibited in the dividing cells. Although the factor(s) that mediates the nuclear export of late mRNAs has not been identified, candidates include HuR, hnRNA, and ASF/SF2, which are proteins shuttling between the nucleus and cytoplasm (Figure 5C; Carlsson and Schwartz, 2000; Koffa et al., 2000; McPhillips et al., 2004; Zhao et al., 2004).

#### TRANSLATIONAL CONTROL OF LATE GENE EXPRESSION

As the inhibitory mechanism for late gene expression, the involvement of translational inhibition was also reported. Translation efficiency was suppressed with *in vitro* translated RNA containing the late UTR of HPV1, suggesting the late UTR had the potential to inhibit the translation. For the inhibition, poly(A)-binding protein (PABP) was considered to be responsible (Wiklund et al., 2002). It was also reported that poly(C) binding protein 1 and 2 (PCBP-1 and -2) and hnRNP K bound to the L2 coding region of HPV16 mRNA and inhibited the *in vitro* translation (Collier et al., 1998). The rare codon usages found in L1 and L2 might also contribute to the inhibition of late gene translation (Gu et al., 2004). In terminally differentiated cells, the altered expression ratios of tRNA species could compensate for the inhibitory effect of the rare codon usages (Fang et al., 2007).

#### REGULATION OF VIRAL DNA REPLICATION

E1 and E2 have essential roles in the HPV genome's replication (Kadaja et al., 2009). E2 is a DNA-binding protein that recognizes E2-binding sites (E2BSs) located in the LCR (Figure 3). E2 has transcriptional transactivator activity, as well as the capacity to bind to the viral DNA replication factor E1. E1 has DNA helicase and ATPase activities and weak DNA-binding capacity. Through its interaction with E2, E1 is recruited to the replication origin (ori), which is essential for the initiation of viral DNA replication (Chiang et al., 1992a,b). E2 also contributes to the segregation of viral DNA in the cell division process by tethering the viral DNA to the host chromosome through interaction with Brd4 and/or ChlR1, both of which can bind to chromatin (McBride et al., 2006). Accurate segregation of the viral genome is essential to maintain the HPV infection in the basal cells, in which the copy number of the viral genome is very low.

The replication potential of E1 is regulated by its interaction with cellular proteins. p56, one of the interferon stimulated genes (ISGs), directly interacts with E1 and translocates it to the cytoplasm, which might contribute to the interferon-mediated inhibition of HPV replication (Terenzi et al., 2008). The interaction of WD repeat protein p80 (WDR80) with E1 is reported to be required for the efficient maintenance of the viral genome in undifferentiated keratinocytes (Côté-Martin et al., 2008).

As mentioned, the expression levels of E1 and E2 increase in association with the differentiation of the epithelial cells, which could be responsible for the vegetative genome amplification.

Recently, it was reported that E6 and E7 activated the ATM DNA damage pathway in differentiation-dependent manner, by which Chk2 was activated. Chk2 potentiated caspase-3 and -7, and the caspases in turn cleaved the E1 protein, which might be required for viral DNA amplification in the differentiated cells (Moody et al., 2007; Moody and Laimins, 2009).

Nuclear accumulation of E1 blocks cell cycle progression in early S-phase and triggers the activation of a DNA damage response (DDR) and of the ATM pathway (Fradet-Toucotte et al., 2011), and the activation of DDR might facilitate HPV DNA replication (Sakakibara et al., 2011). The nuclear accumulation of E1 is regulated by phosphorylation of the nuclear export signal (NES) found in E1 via Cyclin E/A-Cdk2 (Fradet-Toucotte et al., 2010).

DNA replication of HPV utilizes the replication mode with a “ $\theta$ -structure” in undifferentiated cells, and the mode changes to “rolling circle replication” in differentiated cells, which enables the rapid synthesis of multiple copies of viral DNA. The molecular mechanism supporting the DNA replication in the differentiated cells, however, has not been fully explained (Flores and Lambert, 1997).

#### INVOLVEMENT OF CELLULAR TRANSCRIPTION FACTORS IN VIRAL DNA REPLICATION

It was reported that the binding of hSkn-1a to its recognition site proximal to the ori region was required for the viral genome replication of HPV16 (Kukimoto et al., 2008). In other HPV types, Sp1 and TATA box binding protein (TBP) inhibit viral genome replication, in which competition between E2 and Sp1 or TBP may be involved (Demeret et al., 1995; Hartley and Alexander, 2002). These transcription factors might alter the chromatin structure, which could inhibit the access of E1 to the origin (Demeret et al., 1995). The inhibition of STAT-1 expression by E6 and E7 was also reported to be important for viral genome amplification (Hong et al., 2011).

#### VIRION PRODUCTION

A report described that HSP70 was activated in response to the keratinocyte differentiation and co-localized with HPV31 L1 in the differentiated layer of epithelium (Song et al., 2010). It was reported that the disulfide bond among the HPV16 L1 pentamer was formed in a differentiation-dependent manner and had an important role in virion stability (Conway et al., 2011), indicating that virion production was regulated not only by the quantity of the late gene products but also by a differentiation-dependent mechanism.

#### THE MODULATION OF CELL PROLIFERATION/DIFFERENTIATION BY HPV GENE PRODUCTS

As described above, HPV replication is strictly regulated by the differentiation program of the host cells. Conversely the HPV infections modulate the proliferation/differentiation status of the host cells, indicating tight communication between the virus and the host cell, which is required for completion of the viral replication.

#### ACCELERATION OF CELL PROLIFERATION AND INHIBITION OF CELL DIFFERENTIATION

The acceleration of cell proliferation and inhibition of differentiation are induced by the expression of E6 and E7 (Longworth and Laimins, 2004; Moody and Laimins, 2010). E7 inhibits the functions of the pocket protein family, activates the E2F-dependent promoter, and induces S-phase-specific gene expression (Moody and Laimins, 2010). E7 maintains Cyclin E or Cyclin A-CDK2 activity in differentiated cells by targeting p21 and p27, important regulators for growth arrest in the differentiation process. E6 mediates ubiquitination of p53 in association with E6AP, causing the proteasomal degradation of p53 (Moody and Laimins, 2010), and disturbs p53-mediated growth arrest. The association between E6 and histone acetyltransferases (HATs) might be also involved in the inhibition of p53 function (Moody and Laimins, 2010). E6 was reported to target cellular PDZ proteins, which might contribute to the immortalization of the infected cells (Thomas et al.,

2008). E6 was reported to activate telomerase activity by inducing the overexpression of c-Myc and by modulating the expression of NFX1-91, which also contributed to the immortalization process (Gewin et al., 2004).

The functions of E6 and E7 in the activation of the DNA replication machinery of the host cell are required to ensure the viral genome's replication in the differentiated cells (Thomas et al., 1999), resulting in the aberrant proliferation and the retarded differentiation of the host cells. With a transgenic mouse model expressing HPV16 E6 and/or E7 under the K14 promoter, dysplasia was observed at the stratified epidermis (Griep et al., 1993).

Although the normal differentiation of keratinocytes is not fully understood, reports describe the involvement of Notch, MAPK, NF- $\kappa$ B, p63, the AP2 family, C/EBP, IRF6, GRHL3, and KLF4 (Blanpain and Fuchs, 2009). There are also papers describing the contribution of c-Myc to the differentiation of epithelial stem cells, and differentiation-dependent demethylation at histone H3K27 (Blanpain and Fuchs, 2009). Recently, some of these factors were found to be associated with HPV gene products (Lathion et al., 2003; Chakrabarti et al., 2004; An et al., 2008; Melar-New and Laimins, 2010), and it is reasonable that these associations modify the cell differentiation program of the infected cells.

#### INHIBITION OF THE APOPTOTIC INDUCTION

The aberrant proliferation and/or DNA replication in the HPV-infected cells induce p53-dependent apoptotic cell death. The inactivation of pRb by E7 also potentiates the p53 activity. The p53-dependent apoptosis is counteracted by E6 activity (Garnett et al., 2006; Moody and Laimins, 2010). E6 induces the proteasomal degradation of p53. It has also been reported that E6 directly binds to p53 and inhibits its DNA-binding potential (Lechner and Laimins, 1994), and that E6 interacts with HDAC p300, ADA3, and/or CREB-binding protein (CBP), which destabilizes p53 (Patel et al., 1999; Zimmermann et al., 1999; Kumar et al., 2002). The HPV-infected cells also escaped from anoikis by the E6-mediated expression of FAK and the phosphorylation of paxillin, which activates FAK (McCormack et al., 1997). It was reported that interaction between E7 and p600 was involved in the inhibition of anoikis (Huh et al., 2005).

Several membrane-spanning death receptors have been identified, such as TNF receptor type 1 (TNFR1), FAS receptor, and TRAIL receptor. The high-risk type E6 was reported to interact with TNFR1 and suppress TNF $\alpha$ -induced apoptosis (Filippova et al., 2002). E6 is also known to interact with FADD and caspase8, which might inhibit the apoptosis mediated by FAS and TRAIL (Filippova et al., 2004; Garnett et al., 2006). In addition, E6 was reported to be associated with pro-apoptotic Bcl2 members, BAK and BAX, and to interfere with intrinsic apoptosis (Garnett et al., 2006). It was reported that E5 could inhibit FAS- and TRAIL-mediated apoptosis (Garnett et al., 2006). In addition, there was a paper that described the inhibitory effect of E7 on apoptosis, in which the upregulation of cellular inhibitor of apoptosis protein (c-IAP) by E7 was involved (Garnett et al., 2006).

#### THE MODULATION OF miRNA EXPRESSION THROUGH CELL DIFFERENTIATION

Recently, it was reported that HPV E6 and E7 induced the aberrant expression of tumor suppressive miRNAs (Zheng and Wang,

2011). E6 and E7 are known to target c-Myc, p53, and E2F, and these transcription factors are reported to be involved in the regulation of miRNA expression, so E6 and E7 could cause the uncoordinated expression of those miRNAs. E6 and E7 target p53 and pRb, respectively, and cause the unregulated expression of the miR-15/16 cluster, the miR-17-92 family, miR-21, miR-23b, miR-34a, and the miR-106b/93/25 cluster. Such aberrant expression was suspected to be involved in the development of cervical cancer. It was also reported that E6, E7, and E5 suppress the expression of miR-203 which participates in the differentiation of epithelial cells (McKenna et al., 2010; Greco et al., 2011). Through the suppression of miR-203, the expression level of p63 is upregulated in the differentiated cells, which might contribute to the genome amplification and the late gene expression in the upper layers of epithelium (Melar-New and Laimins, 2010).

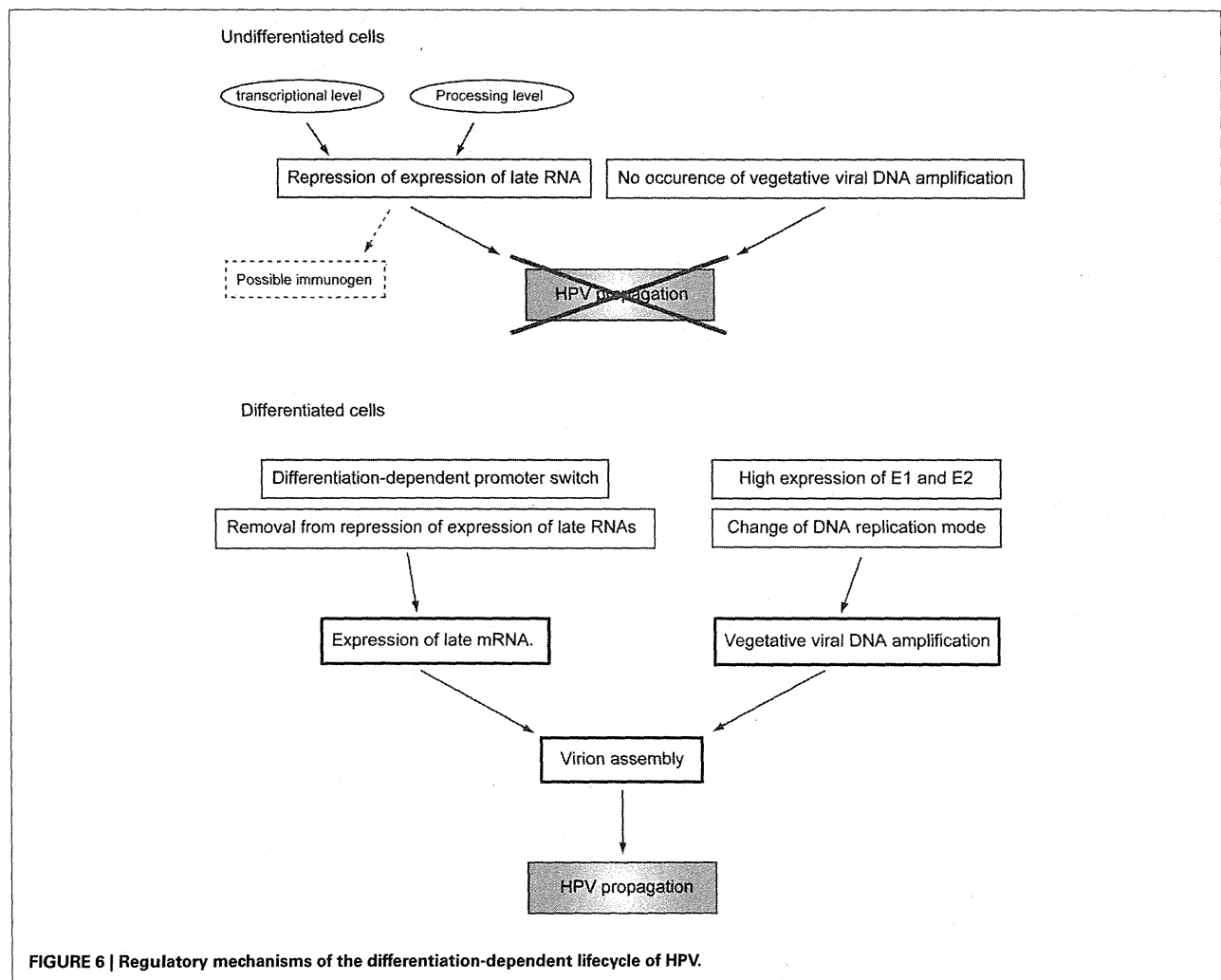
#### EPIGENETIC ALTERATION INDUCED BY THE HPV INFECTION

There was a report describing the epigenetic alteration of cells mediated by HPV gene expression (Hsu et al., 2011; Hyland et al., 2011; McLaughlin-Drubin et al., 2011; Zheng and Wang,

2011). The epigenetic alteration induced by the HPV infection was considered to modify the miRNA expression pattern, which might change the cell differentiation program. Although there is a possibility that some epigenetic alteration occurs also in the normal cell differentiation process, there has been no report related to it.

#### CONCLUSION

Human papillomavirus suppresses its replication to a “maintenance level” or “latent infection mode” in the basal cells, and maintains the DNA synthesis potential of the infected cells detached from the basal membrane to maintain viral genome replication. In terminally differentiated cells, a tremendous level of genome amplification and late gene expression takes place. After completion of virion assembly, the virions are released externally with the cornified cells (the regulation of the differentiation-dependent lifecycle of HPV is summarized in **Figure 6**). One of the reasons for this unique lifecycle of HPV is escape from the immune-surveillance system (Bodily and Laimins, 2011). Because both L1 and L2 could have immunogenicity, their expressions should be suppressed until the infected cells move to the upper layer of the



epithelium, where the host immune-surveillance system does not well function. The hyperproliferation induced by E6 and E7 is required for viral genome amplification and contributes to the augmentation of progeny virion production by expanding the pool of the infected cells.

Tight communication between the virus and the host cell is a unique character of HPV biology, and raises the possibility of using HPV as a probe to investigate the development of the stratified epithelium. In this review, we did not describe the details of the cancer progression induced by HPV infections.

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## Genotype Distribution of Human Papillomaviruses in Japanese Women with Abnormal Cervical Cytology

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**Abstract:** We report the prevalence and genotype distribution of human papillomaviruses (HPVs) among Japanese women with abnormal cervical cytology using the PGMY-CHUV assay, one of PGMY-PCR-based lineblot assays that was validated and shown to be suitable for the detection of multiple HPV types in a specimen with minimum bias. Total DNA was extracted from cervical exfoliated cells collected from 326 outpatients with abnormal Pap smears. Overall, 307 specimens (94%) were HPV-positive, 30% of which contained multiple genotypes. The prevalence of HPV DNA was 83% (49/59 samples) in atypical squamous cells of undetermined significance (ASC-US); 91% (20/22 samples) in atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion (ASC-H); 97% (130/134 samples) in low-grade squamous intraepithelial lesion (LSIL); and 99% (85/86 samples) in high-grade squamous intraepithelial lesion (HSIL). Three most frequent HPV types detected in HSIL were HPV16 (36%), HPV52 (24%), and HPV58 (14%). Our results suggest that multiple HPV infections are more prevalent in Japanese women than previously reported, and confirm that HPV52 and 58 are more dominant in their cervical precancerous lesions when compared to those reported in Western countries.

**Keywords:** Human papillomavirus, HPV genotyping, cervical cancer, Pap smear, abnormal cytology, HPV vaccine.

### INTRODUCTION

Human papillomaviruses (HPVs) are the causative agents of cervical cancer, cervical intraepithelial neoplasia, and other anogenital cancers [1]. Among more than 100 HPV types so far identified, nearly 40 types infecting the anogenital mucosa are classified as either low- or high-risk types on the basis of their oncogenic potential [2]. A previous large-scale epidemiological study identified 15 HPV types, HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82, which are closely linked to the development of cervical cancer [3], with approximately 70% of cervical cancer cases worldwide attributed to HPV16 and 18 [4].

Two prophylactic HPV vaccines, one quadrivalent vaccine directed against HPV6, 11, 16, and 18 [Gardasil<sup>®</sup> (Merck and Co., Whitehouse Station, NJ, USA)], and one bivalent vaccine against HPV16 and 18 [Cervarix<sup>®</sup> (GlaxoSmithKline Biologicals, Rixensart, Belgium)], are now being introduced worldwide. Both vaccines exhibit a high efficacy for preventing cervical precancerous lesions caused by vaccine-targeted HPV types in clinical trials for

HPV-uninfected women [5,6]. In Japan, the bivalent vaccine was first approved for use in 2009, followed by the quadrivalent vaccine in 2011, both being introduced with the expectation of reducing cervical cancer cases. However, previous epidemiological studies performed in Japanese women have shown variation in the proportion of HPV16 and 18 in cervical cancer, ranging from 50 to 70% [7-9], which makes it difficult to predict the real impact of the HPV vaccination on cervical cancer prevention in the Japanese population.

Because currently available HPV vaccines are thought to be effective only for the vaccine-targeted types, precise determination of the HPV genotype distribution in cervical cancer and its precancerous lesions is needed for assessment of the vaccine efficacy and planning of future vaccination strategies. In 2006, the World Health Organization (WHO) established the HPV laboratory network (LabNet) with the aim of harmonizing and standardizing laboratory testing procedures to promote consistent laboratory evaluation of HPV-related disease burden and monitoring of the performance of HPV vaccines. Through a series of collaborative studies for standardizing HPV genotyping assays, the LabNet has recently described a PGMY-lineblot assay (PGMY-CHUV [10]) as a reliable, low-cost HPV genotyping method [11,12]. The PGMY-CHUV assay consists of PCR with biotinylated generic PGMY09/11

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primers to amplify the HPV L1 region and subsequent reverse-blotting hybridization with type-specific DNA probes, and is suitable to detect multiple HPV types in a specimen with minimum bias [10,13]. Most studies on the HPV genotype distribution in Japanese women, however, have relied on PCR of HPV DNA using a limited pair of L1 consensus primers, such as LC1/LC2 [7,14-20], which is less sensitive to detect multiple-genotypes infection compared to other PCR assays using type-specific primers, which might have resulted in less confident data on HPV prevalence in Japanese women.

In this study we examined the prevalence and distribution of HPV genotypes among Japanese women with abnormal Pap smears by using the PGMY-CHUV assay. The data indicate that multiple HPV infections are more prevalent in Japanese women than previously reported, and confirm that HPV52 and 58 are more dominant in cervical precancerous lesions of Japanese women than in those of Western countries' women.

## MATERIALS AND METHODOLOGY

### Study Population and Specimen Collection

From November 2009 to May 2011, we enrolled 1088 women who visited NTT Medical Center Tokyo, a regional medical center in Tokyo, as outpatients having some sort of symptoms or referred by primary care physicians for further examination. The women were subjected to the Papanicolaou (Pap) smear test, leading to diagnoses of 762 (70%) negative for intraepithelial lesion or malignancy (NILM) and 326 (30%) abnormal cytology cases, based on the criteria of the Bethesda System 2001 (Fig. 1). Together with sampling Pap smears, cervical exfoliated cells were collected in Thinprep® collection media by using a Cervex-brush® combi for HPV genotyping. The women with abnormal cytology were further examined by colposcopy, and tissue specimens obtained by punch biopsy were histologically diagnosed. Among 86 HSIL cases, 53 cases were treated by cervical conization, and excised tissues were finally histologically

diagnosed as 1 CIN1, 9 CIN2, and 42 CIN3 cases (Fig. 1). All ICC cases diagnosed by the Pap test were finally histologically confirmed by tissue biopsy. The study protocol was approved by the Ethics Committee at NTT Medical Center Tokyo, and written informed consent for study participation was obtained from each patient.

### HPV DNA Detection and Genotyping

Total DNA was extracted from a 200- $\mu$ L aliquot of the suspended cell samples by using the QIAamp DNA Blood Mini Kit (Qiagen), resulting in the final elution volume of 100  $\mu$ L Tris-EDTA buffer. Cross-contamination between samples during DNA extraction was evaluated by simultaneous extraction from only a collection medium. A 3- $\mu$ L aliquot of the purified DNA was then used for PCR amplification with AmpliTaq Gold® polymerase (GE Healthcare Bio-Sciences), biotinylated PGMY09/11 primers, to amplify the L1 DNA of >40 HPV genotypes, and biotinylated HLA primers, to amplify cellular HLA DNA. Positive control (0.1 pg/mL of HPV16 DNA as a plasmid) and negative control (dH<sub>2</sub>O) were included in every 50 PCR reactions to verify the sensitivity of PCR and monitor contamination of HPV DNA in reagents. The PCR products were run on 1.5% agarose gels to assign the positivity of HPV DNA amplification and to confirm the integrity of the extracted DNA as amplification of HLA DNA. Reverse blotting hybridization was done as described in the Human Papillomavirus Laboratory Manual published by the WHO [11]. Briefly, 15- $\mu$ L denatured PCR products were allowed to hybridize with oligonucleotide probes specific for 31 HPV types (HPV6, 11, 16, 18, 26, 31, 33, 34, 35, 39, 40, 42, 44, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 66, 68, 69, 70, 73, 82, 83, and 84) immobilized on a Biodyne C membrane (Pall corporation) using a Miniblotter MN45 (Immunitics, Cambridge, MA, USA). The hybridized DNA was detected using streptavidin-HRP (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and ECL detection reagent (GE Healthcare Bio-Sciences). After reading positive signals, PCR-positive but hybridization-negative samples were

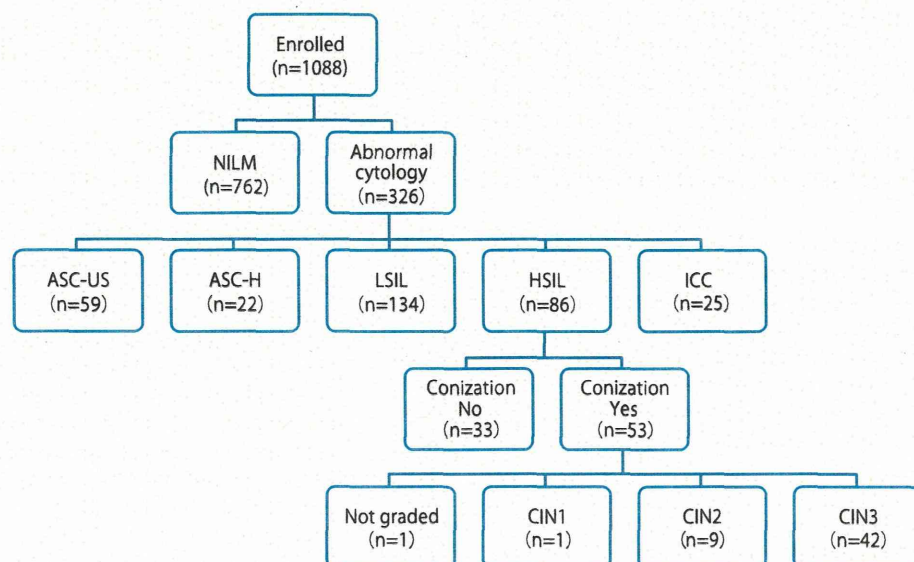


Fig. (1). Overview of study population. n indicates numbers of patients.