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^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

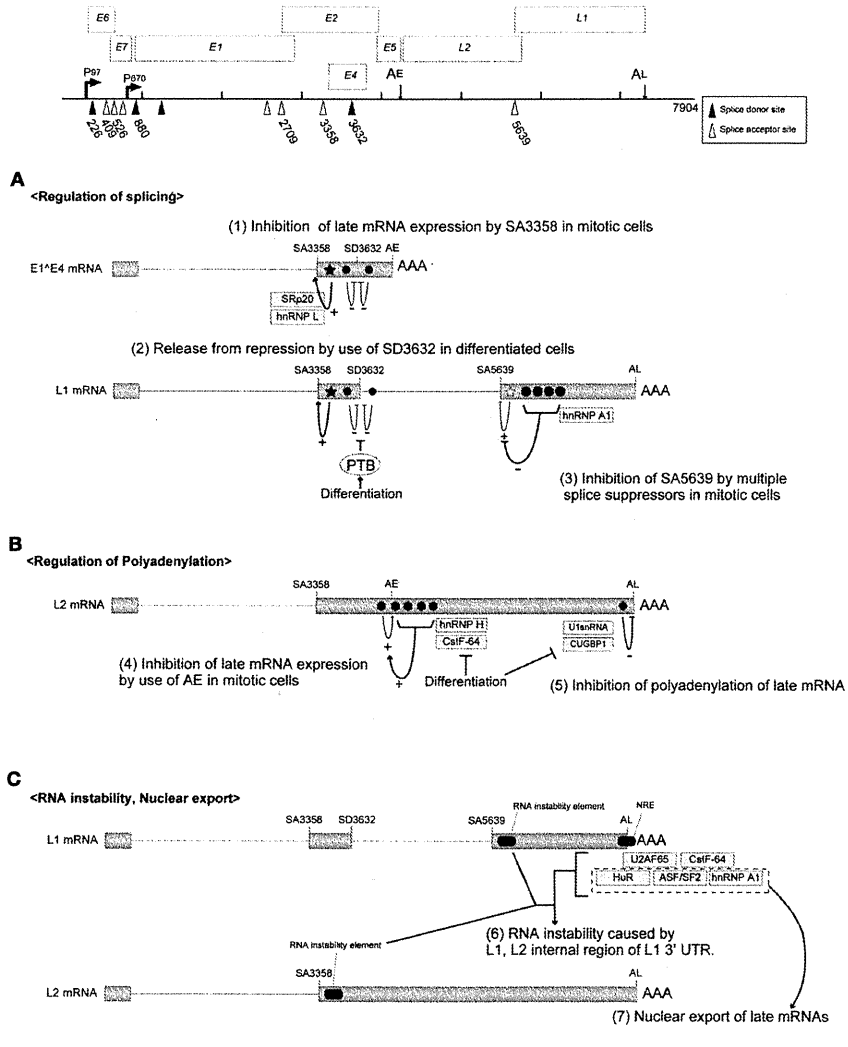

FIGURE 5 | Regulatory mechanisms for mRNA processing. The HPV16 genome with the promoters, the poly(A) signals, and the splicing signals are shown at the top of the figure. Open triangles indicate splicing acceptors and filled triangles indicate splicing donors. **(A)** HPV16 splicing regulatory elements are indicated as filled stars (splicing enhancers) or filled circles (splicing silencers). **(B)** HPV16 polyadenylation regulatory elements are indicated as filled hexagons (polyadenylation stimulatory elements) and a filled rectangle (polyadenylation inhibitory element). **(C)** The regulatory elements for the instability and nuclear export of HPV16 late mRNA are indicated. This figure is cited from a review (Schwartz, 2008).

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 "θ-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 (Grieg et al., 1993).

Although the normal differentiation of keratinocytes is not fully understood, reports describe the involvement of Notch, MAPK, NF-κB, 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 TNFα-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

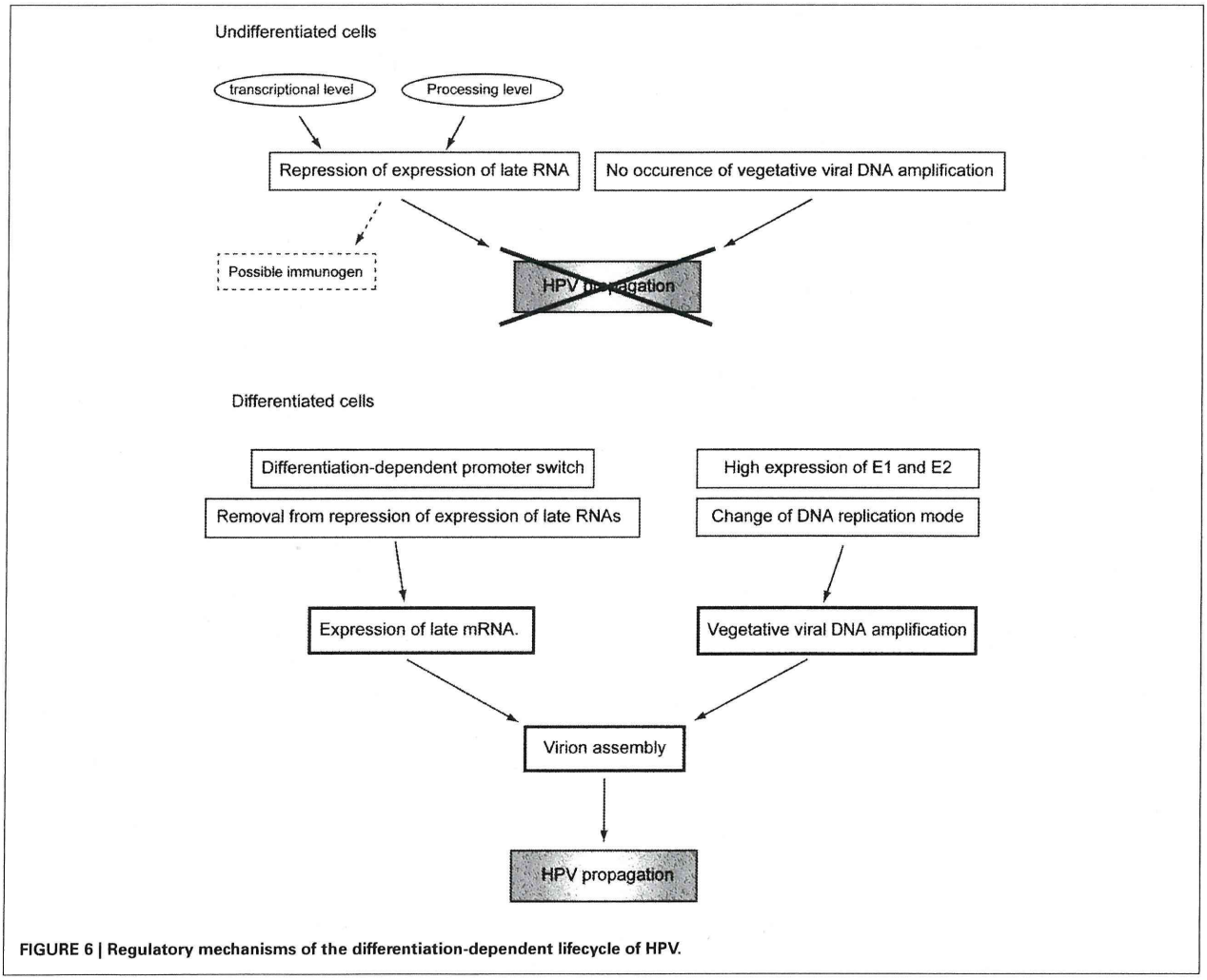

FIGURE 6 | Regulatory mechanisms of the differentiation-dependent lifecycle of HPV.

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. HPV-induced cancer is a good model for "multi-step carcinogenesis," and the study of HPV biology provides novel insights into cancer development.

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REFERENCES

Ai, W., Narahari, J., and Roman, A. (2000). Yin yang 1 negatively regulates the differentiation-specific E1 promoter of human papillomavirus type 6. *J. Virol.* 74, 5198–5205.

Ai, W., Toussaint, E., and Roman, A. (1999). CCAAT displacement protein binds to and negatively regulates human papillomavirus type 6 E6, E7, and E1 promoters. *J. Virol.* 73, 4220–4229.

An, J., Mo, D., Liu, H., Veena, M. S., Srivatsan, E. S., Massoumi, R., and Rettig, M. B. (2008). Inactivation of the CYLD deubiquitinase by HPV E6 mediates hypoxia-induced NF-kappaB activation. *Cancer Cell* 14, 394–407.

Apt, D., Watts, R. M., Suske, G., and Bernard, H. U. (1996). High Sp1/Sp3 ratios in epithelial cells during epithelial differentiation and cellular transformation correlate with the activation of the HPV-16 promoter. *Virology* 224, 281–291.

Barreau, C., Paillard, L., and Osborne, H. B. (2005). AU-rich elements and associated factors: are there unifying principles? *Nucleic Acids Res.* 33, 7138–7150.

Bedell, M. A., Hudson, J. B., Golub, T. R., Turyk, M. E., Hosken, M., Wilbanks, G. D., and Laimins, L. A. (1991). Amplification of human papillomavirus genomes in vitro is dependent on epithelial differentiation. *J. Virol.* 65, 2254–2260.

Blanpain, C., and Fuchs, E. (2009). Epidermal homeostasis: a balancing act of stem cells in the skin. *Nat. Rev. Mol. Cell Biol.* 10, 207–217.

Bodily, J., and Laimins, L. A. (2011). Persistence of human papillomavirus infection: keys to malignant progression. *Trends Microbiol.* 19, 33–39.

Bodily, J. M., Mehta, K. P., Cruz, L., Meyers, C., and Laimins, L. A. (2011). The E7 open reading frame acts in cis and in trans to mediate differentiation-dependent activities in the human papillomavirus type 16 life cycle. *J. Virol.* 85, 8852–8862.

Carlsson, A., and Schwartz, S. (2000). Inhibitory activity of the human papillomavirus type 1 AU-rich element correlates inversely with the levels of the elav-like HuR protein in the cell cytoplasm. *Arch. Virol.* 145, 491–503.

Carter, J. R., Ding, Z., and Rose, B. R. (2011). HPV infection and cervical disease: a review. *Aust. N. Z. J. Obstet. Gynaecol.* 51, 103–108.

Chakrabarti, O., Veeraraghavalu, K., Tergaonkar, V., Liu, Y., Androphy, E. J., Stanley, M. A., and Krishna, S. (2004). Human papillomavirus type 16 E6 amino acid 83 variants enhance E6-mediated MAPK signaling and differentially regulate tumorigenesis by notch signaling and oncogenic Ras. *J. Virol.* 78, 5934–5945.

Cheunim, T., Zhang, J., Milligan, S. G., McPhillips, M. G., and Graham, S. V. (2008). The alternative splicing factor hnRNP A1 is up-regulated during virus-infected epithelial cell differentiation and binds the human papillomavirus type 16 late regulatory element. *Virus Res.* 131, 189–198.

Chiang, C. M., Dong, G., Broker, T. R., and Chow, L. T. (1992a). Control of human papillomavirus type 11 origin of replication by the E2 family of transcription regulatory proteins. *J. Virol.* 66, 5224–5231.

Chiang, C. M., Ustav, M., Stenlund, A., Ho, T. F., Broker, T. R., and Chow, L. T. (1992b). Viral E1 and E2 proteins support replication of homologous and heterologous papillomaviral origins. *Proc. Natl. Acad. Sci. U.S.A.* 89, 5799–5803.

Collier, B., Goobar-Larsson, L., Sokolowski, M., and Schwartz, S. (1998). Translational inhibition in vitro of human papillomavirus type 16 L2 mRNA mediated through interaction with heterogeneous ribonucleoprotein K and poly(rC)-binding proteins 1 and 2. *J. Biol. Chem.* 273, 22648–22656.

Collier, B., Oberg, D., Zhao, X., and Schwartz, S. (2002). Specific inactivation of inhibitory sequences in the 5′ end of the human papillomavirus type 16 L1 open reading frame results in production of high levels of L1 proteins in human epithelial cells. *J. Virol.* 76, 2739–2752.

Conway, M. J., Cruz, L., Alam, S., Christensen, N. D., and Meyers, C. (2011). Differentiation-dependent interpentameric disulfide bond stabilizes native human papillomavirus type 16. *PLoS ONE* 6, e22427. doi:10.1371/journal.pone.0022427

Côté-Martin, A., Moody, C., Fradet-Turcotte, A., D'Abramo, C. M., Lehoux, M., Joubert, S., Poirier, G. G., Coulombe, B., Laimins, L. A., and Archambault, J. (2008). Human papillomavirus E1 helicase interacts with the WD repeat protein p80 to promote maintenance of the viral genome in keratinocytes. *J. Virol.* 82, 1271–1283.

Demeret, C., Le Moal, M., Yaniv, M., and Thierry, F. (1995). Control of HPV 18 DNA replication by cellular and viral transcription factors. *Nucleic Acids Res.* 23, 4777–4784.

Desaintes, C., and Demeret, C. (1996). Control of papillomavirus DNA replication and transcription. *Semin. Cancer Biol.* 7, 339–347.

Dietrich-Goetz, W., Kennedy, I. M., Levins, B., Stanley, M. A., and Clements, J. B. (1997). A cellular 65-kDa protein recognizes the negative regulatory element of human papillomavirus late mRNA. *Proc. Natl. Acad. Sci. U.S.A.* 94, 163–168.

Dürst, M., Glitz, D., Schneider, A., and zur Hausen, H. (1992). Human papillomavirus type 16 (HPV 16) gene expression and DNA replication in cervical neoplasia: analysis by in situ hybridization. *Virology* 189, 132–140.

Fang, L., Budgeon, L. R., Doorbar, J., Briggs, E. R., and Howett, M. K. (2006). The human papillomavirus type 11 E1/\E4 protein is not essential for viral genome amplification. *Virology* 351, 271–279.

Fang, N. X., Gu, W., Ding, J., Saunders, N. A., Frazer, I. H., and Zhao, K. N. (2007). Calcium enhances mouse keratinocyte differentiation in vitro to differentially regulate expression of papillomavirus authentic and codon modified L1 genes. *Virology* 365, 187–197.

Fehrmann, F., Klumpp, D. J., and Laimins, L. A. (2003). Human papillomavirus type 31 E5 protein supports cell cycle progression and activates late viral functions upon epithelial differentiation. *J. Virol.* 77, 2819–2831.

Filippova, M., Parkhurst, L., and Duerksen-Hughes, P. J. (2004). The human papillomavirus 16 E6 protein binds to Fas-associated death domain and protects cells from Fas-triggered apoptosis. *J. Biol. Chem.* 279, 25729–25744.

Filippova, M., Song, H., Connolly, J. L., Dermody, T. S., and Duerksen-Hughes, P. J. (2002). The human papillomavirus 16 E6 protein binds to tumor necrosis factor (TNF) R1 and protects cells from TNF-induced apoptosis. *J. Biol. Chem.* 277, 21730–21739.

Flores, E. R., and Lambert, P. F. (1997). Evidence for a switch in the mode of human papillomavirus type 16 DNA replication during the viral life cycle. *J. Virol.* 71, 7167–7179.

Fradet-Toucotte, A., Bergeron-Labrecque, F., Moody, C. A., Lehoux, M., Laimins, L. A., and Archambault, J. (2011). Nuclear accumulation of the papillomavirus E1 helicase blocks S-phase progression and triggers an ATM-dependent DNA damage response. *J. Virol.* 85, 8996–9012.

Fradet-Toucotte, A., Moody, C. A., Laimins, L. A., and Archambault, J. (2010). Nuclear export of human papillomavirus type 31 E1 is regulated by cdk2 phosphorylation and required for viral genome maintenance. *J. Virol.* 84, 11747–11760.

Frattini, M. G., Lim, H. B., and Laimins, L. A. (1996). In vitro synthesis of oncogenic human papillomaviruses requires episomal genomes for differentiation-dependent late expression. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3062–3067.

Furth, P. A., Choe, W. T., Rex, J. H., Byrne, J. C., and Baker, C. C. (1994). Sequences homologous to 5′ splice sites are required for the inhibitory activity of papillomavirus late 3′ untranslated regions. *Mol. Cell. Biol.* 14, 5278–5289.

Garnett, T. O., Filippova, M., and Duerksen-Hughes, P. J. (2006). Accelerated degradation of FADD and procaspase 8 in cells expressing human papillomavirus 16 E6 impairs TRAIL-mediated apoptosis. *Cell Death Differ.* 13, 1915–1926.

Genther, S. M., Sterling, S., Duensing, S., Münger, K., Sattler, C., and Lambert, P. F. (2003). Quantitative role of the human papillomavirus type 16 E5 gene during the productive stage of the viral life cycle. *J. Virol.* 77, 2832–2842.

Gewin, L., Myers, H., Kiyono, T., and Galloway, D. A. (2004). Identification of a novel telomerase repressor that interacts with the human papillomavirus type-16 E6/E6-AP complex. *Genes Dev.* 18, 2269–2282.

Goraczniak, R., and Gunderson, S. I. (2008). The regulatory element in the 3′-untranslated region of human papillomavirus 16 inhibits expression by binding CUG-binding protein 1. *J. Biol. Chem.* 283, 2286–2296.

Greco, D., Kivi, N., Qian, K., Leivonen, S. K., Auvinen, P., and Auvinen, E. (2011). Human papillomavirus 16 E5 modulates the expression of host microRNAs. *PLoS ONE* 6, e21646. doi:10.1371/journal.pone.0021646

Griep, A. E., Herber, R., Jeon, S., Lohse, J. K., Dubielzig, R. R., and Lambert, P. F. (1993). Tumorigenicity by human papillomavirus type 16 E6 and E7 in transgenic mice correlates with alterations in epithelial cell growth and differentiation. *J. Virol.* 67, 1373–1384.

Gu, W., Li, M., Zhao, W. M., Fang, N. X., Bu, S., Frazer, I. H., and Zhao, K. N. (2004). tRNASer(CGA) differentially regulates expression of wild-type and codon-modified papillomavirus L1 genes. *Nucleic Acids Res.* 32, 4448–4461.

Hadaschik, D., Hinterkeuser, K., Oldak, M., Pfister, H. J., and Smola-Hess, S. (2003). The papillomavirus E2 protein binds to and synergizes with C/EBP factors involved in keratinocyte differentiation. *J. Virol.* 77, 5253–5265.

Hartley, K. A., and Alexander, K. A. (2002). Human TATA binding protein inhibits human papillomavirus type 11 DNA replication by antagonizing E1-E2 protein complex formation on the viral origin of replication. *J. Virol.* 76, 5014–5023.

Hong, S., Mehta, K. P., and Laimins, L. A. (2011). Suppression of STAT-1 expression by human papillomaviruses is necessary for differentiation-dependent genome amplification and plasmid maintenance. *J. Virol.* 85, 9486–9494.

Howley, M. P. (1996). "Papillomavirinae: the viruses and their replication," in *Fields Virology*, 3rd Edn, eds B. N. Fields, D. M. Knipe, and P. M. Howley (Philadelphia: Lippincott Williams and Wilkins), 2045–2076.

Hsu, C. H., Peng, K. L., Jhang, H. C., Lin, C. H., Wu, S. Y., Chiang, C. M., Lee, S. C., Yu, W. C., and Juan, L. J. (2011). The HPV E6 oncoprotein targets histone methyltransferases for modulating specific gene transcription. *Oncogene.*

Huh, K. W., DeMasi, J., Ogawa, H., Nakatani, Y., Howley, P. M., and Münger, K. (2005). Association of the human papillomavirus type 16 E7 oncoprotein with the 600-kDa retinoblastoma protein-associated factor, p600. *Proc. Natl. Acad. Sci. U.S.A.* 102, 11492–11497.

Hummel, M., Hudson, J. B., and Laimins, L. A. (1992). Differentiation-induced and constitutive transcription of human papillomavirus type 31b in cell lines containing viral episomes. *J. Virol.* 66, 6070–6080.

Hyland, P. L., McDade, S. S., McCloskey, R., Dickson, G. J., Arthur, K., McCance, D. J., and Patel, D. (2011). Evidence for alteration of EZH2, BMI1, and KDM6A and epigenetic reprogramming in human papillomavirus type 16 E6/E7-expressing keratinocytes. *J. Virol.* 85, 10999–11006.

Jeon, S., and Lambert, P. F. (1995). Integration of human papillomavirus type 16 DNA into the human genome leads to increased stability of E6 and E7 mRNAs: implications for cervical carcinogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 92, 1654–1658.

Jia, R., Liu, X., Tao, M., Kruhlak, M., Guo, M., Meyers, C., Baker, C. C., and Zheng, Z. M. (2009). Control of the papillomavirus early-to late switch by differentially expressed SRp20. *J. Virol.* 83, 167–180.

Jones, P. H., Simons, B. D., and Watt, F. M. (2007). Sic transit gloria: farewell to the epidermal transit amplifying cell? *Cell Stem Cell* 1, 371–381.

Kadaja, M., Isok-Paas, H., Laos, T., Ustav, E., and Ustav, M. (2009). Mechanism of genomic instability in cells infected with the high-risk human papillomaviruses. *PLoS Pathog.* 5, e1000397. doi:10.1371/journal.ppat.1000397

Kennedy, I. M., Haddow, J. K., and Clements, J. B. (1991). A negative regulatory element in the human papillomavirus type 16 genome acts at the level of late mRNA stability. *J. Virol.* 65, 2093–2097.

Kim, K., Garner-Hamrick, P. A., Fisher, C., Lee, D., and Lambert, P. F. (2003). Methylation patterns of papillomavirus DNA, its influence on E2 function, and implications in viral infection. *J. Virol.* 77, 12450–12459.

Kines, R. C., Thompson, C. D., Lowy, D. R., Schiller, J. T., and Day, P. M. (2009). The initial steps leading to papillomavirus infection occur on the basement membrane prior to cell surface binding. *Proc. Natl. Acad. Sci. U.S.A.* 106, 20458–20463.

Koffa, M. D., Graham, S. V., Takagaki, Y., Manley, J. L., and Clements, J. B. (2000). The human papillomavirus type 16 negative regulatory RNA element interacts with three proteins that act at different posttranscriptional levels. *Proc. Natl. Acad. Sci. U.S.A.* 97, 4677–4682.

Kukimoto, I., and Kanda, T. (2001). Displacement of YY1 by differentiation-specific transcription factor hSkn-1a activates the P(670) promoter of human papillomavirus type 16. *J. Virol.* 75, 9302–9311.

Kukimoto, I., Mori, S., Sato, H., Takeuchi, T., and Kanda, T. (2008). Transcription factor human Skn-1a enhances replication of human papillomavirus DNA through the direct binding to two sites near the viral replication origin. *FEBS J.* 275, 3123–3135.

Kumar, A., Zhao, Y., Meng, G., Zeng, M., Srinivasan, S., Delmolino, L. M., Gao, Q., Dimri, G., Weber, G. F., Wazer, D. E., Band, H., and Band, V. (2002). Human papillomavirus oncoprotein E6 inactivates the transcriptional coactivator human ADA3. *Mol. Cell. Biol.* 22, 5801–5812.

Lace, M. J., Anson, J. R., Turek, L. P., and Haugen, T. H. (2008). Functional mapping of the human papillomavirus type 16 E1 cistron. *J. Virol.* 82, 10724–10734.

Lathion, S., Schaper, J., Beard, P., and Raj, K. (2003). Notch1 can contribute to viral-induced transformation of primary human keratinocytes. *Cancer Res.* 63, 8687–8694.

Lechner, M. S., and Laimins, L. A. (1994). Inhibition of p53 DNA binding by human papillomavirus E6 proteins. *J. Virol.* 68, 4262–4273.

Longworth, M. S., and Laimins, L. A. (2004). Pathogenesis of human papillomaviruses in differentiating epithelia. *Microbiol. Mol. Biol. Rev.* 68, 362–372.

Mack, D. H., and Laimins, L. A. (1991). A keratinocyte-specific transcription factor, KRF-1, interacts with AP-1 to activate expression of human papillomavirus type 18 in squamous epithelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 88, 9102–9106.

McBride, A. A., Oliveila, J. G., and McPhillips, M. G. (2006). Partitioning viral genomes in mitosis: same idea, different targets. *Cell Cycle* 5, 1499–1502.

McCormack, S. J., Brazinski, S. E., Moore, J. L., Werness, B. A., and Goldstein, D. J. (1997). Activation of the focal adhesion kinase signal transduction pathway in cervical carcinoma cell lines and human genital epithelial cells immortalized with human papillomavirus type 18. *Oncogene* 15, 265–274.

McKenna, D. J., McDade, S. S., Patel, D., and McCance, D. J. (2010). MicroRNA 203 expression in keratinocytes is dependent on regulation of p53 levels by E6. *J. Virol.* 85, 10644–10652.

McLaughlin-Drubin, M. E., Crum, C. P., and Münger, K. (2011). Human papillomavirus E7 oncoprotein induces KDM6A and KDM6B histone demethylase expression and causes epigenetic reprogramming. *Proc. Natl. Acad. Sci. U.S.A.* 108, 2130–2135.

McPhillips, M. G., Oliveira, J. G., Spindler, J. E., Mitra, R., and McBride, A. A. (2006). Brd4 is required for E2-mediated transcriptional activation but not genome partitioning of all papillomavirus. *J. Virol.* 80, 9530–9543.

McPhillips, M. G., Veerapraditsin, T., Cumming, S. A., Karali, D., Milligan, S. G., Boner, W., Morgan, I. M., and Graham, S. V. (2004). SF2/ASF binds the human papillomavirus type 16 late RNA control element and is regulated during differentiation of virus-infected epithelial cells. *J. Virol.* 78, 10598–10605.

Melar-New, M., and Laimins, L. A. (2010). Human papillomaviruses modulate expression of microRNA 203 upon epithelial differentiation to control levels of p63 proteins. *J. Virol.* 84, 5212–5221.

Moody, C. A., Fradet-Turcotte, A., Archambault, J., and Laimins, L. A. (2007). Human papillomaviruses activate caspases upon epithelial differentiation to induce viral genome

amplification. *Proc. Natl. Acad. Sci. U.S.A.* 104, 19541–19546.

Moody, C. A., and Laimins, L. A. (2009). Human papillomaviruses activate the ATM DNA damage pathway for viral genome amplification upon differentiation. *PLoS Pathog.* 5, e1000605. doi:10.1371/journal.ppat.1000605

Moody, C. A., and Laimins, L. A. (2010). Human papillomavirus oncoproteins: pathways to transformation. *Nat. Rev. Cancer* 10, 550–560.

Mori, S., Ozaki, S., Yasugi, T., Yoshikawa, H., Taketani, Y., and Kanda, T. (2006). Inhibitory cis-element-mediated decay of human papillomavirus type 16 L1-transcript in undifferentiated cells. *Mol. Cell. Biochem.* 288, 47–57.

Münger, K., Baldwin, A., Edwards, K. M., Hayakawa, H., Nguyen, C. L., Owens, M., Grace, M., and Huh, K. (2004). Mechanisms of human papillomavirus-induced oncogenesis. *J. Virol.* 78, 11451–11460.

Nakahara, T., Peh, W. L., Doorbar, J., Lee, D., and Lambert, P. F. (2005). Human papillomavirus type 16 E1circumflexE4 contributes to multiple facets of the papillomavirus life cycle. *J. Virol.* 79, 13150–13165.

Oberg, D., Fay, J., Lambkin, H., and Schwartz, S. (2005). A downstream polyadenylation element in human papillomavirus type 16 L2 encodes multiple GGG motifs and interacts with hnRNP H. *J. Virol.* 79, 9254–9269.

Ozbun, M. A., and Meyers, C. (1997). Characterization of late gene transcripts expressed during vegetative replication of human papillomavirus type 31b. *J. Virol.* 71, 5161–5172.

Parkin, D. M., and Bray, F. (2006). Chapter 2: the burden of HPV-related cancers. *Vaccine* 24(Suppl. 3), S3/11–S3/25.

Patel, D., Huang, S. M., Baglia, L. A., and McCance, D. J. (1999). The E6 protein of human papillomavirus type 16 binds to and inhibits co-activation by CBP and p300. *EMBO J.* 18, 5061–5072.

Remm, M., Remm, A., and Ustav, M. (1999). Human papillomavirus type 18 E1 protein is translated from polycistronic mRNA by a discontinuous scanning mechanism. *J. Virol.* 73, 3062–3070.

Rush, M., Zhao, X., and Schwartz, S. (2005). A splicing enhancer in the E4 coding region of human papillomavirus type 16 is required for early mRNA splicing and polyadenylation as well as inhibition of premature late gene expression. *J. Virol.* 79, 12002–12015.

Sakakibara, N., Mitra, R., and McBride, A. A. (2011). The papillomavirus E1 helicase activates a cellular DNA damage response in viral replication foci. *J. Virol.* 85, 8981–8995.

Schwartz, S. (2008). HPV-16 RNA processing. *Front. Biosci.* 13, 5880–5891.

Sokolowski, M., Tan, W., Jellne, M., and Schwartz, S. (1998). mRNA instability elements in the human papillomavirus type 16 L2 coding region. *J. Virol.* 72, 1504–1515.

Somberg, M., Zhao, X., Fröhlich, M., Evander, M., and Schwartz, S. (2008). Polypyrimidine tract binding protein induces human papillomavirus type 16 late gene expression by interfering with splicing inhibitory elements at the major late 5′ splice site, SD3632. *J. Virol.* 82, 3665–3678.

Song, H., Moseley, P. L., Lowe, S. L., and Ozbun, M. A. (2010). Inducible heat shock protein 70 enhances HPV31 viral genome replication and virion production during the differentiation-dependent life cycle in human keratinocytes. *Virus Res.* 147, 113–122.

Steger, G., and Corbach, S. (1997). Dose-dependent regulation of the early promoter of human papillomavirus type 18 by the viral E2 protein. *J. Virol.* 71, 50–58.

Stubenrauch, F., Lim, H. B., and Laimins, L. A. (1998). Differential requirements for conserved E2 binding sites in the life cycle of oncogenic human papillomavirus type 31. *J. Virol.* 72, 1071–1077.

Stünkel, W., and Bernard, H. U. (1999). The chromatin structure of the long control region of human papillomavirus type 16 represses viral oncoprotein expression. *J. Virol.* 73, 1918–1930.

Tan, S. H., Gloss, B., and Bernard, H. U. (1992). During negative regulation of the human papillomavirus-16 E6 promoter, the viral E2 protein can displace Sp1 from a proximal promoter element. *Nucleic Acids Res.* 20, 251–256.

Terenzi, F., Saikia, P., and Sen, G. C. (2008). Interferon-induced protein, p56, inhibits HPV DNA replication by binding to the viral protein E1. *EMBO J.* 27, 3311–3321.

Terhune, S. S., Hubert, W. G., Thomas, J. T., and Laimins, L. A. (2001). Early polyadenylation signals of human papillomavirus type 31 negatively regulate capsid gene expression. *J. Virol.* 75, 8147–8157.

Thierry, F. (2009). Transcriptional regulation of the papillomavirus oncogenes by cellular and viral transcription factors in cervical carcinoma. *Virology* 384, 375–379.

Thomas, J. T., Hubert, W. G., Ruesch, M. N., and Laimins, L. A. (1999). Human papillomavirus type 31 oncoproteins E6 and E7 are required for the maintenance of episomes during the viral life cycle in normal human keratinocytes. *Proc. Natl. Acad. Sci. U.S.A.* 96, 8449–8454.

Thomas, M., Narayan, N., Pim, D., Tomaic, V., Massimi, P., Nagasaka, K., Kranjec, C., Gammoh, N., and Banks, L. (2008). Human papillomaviruses, cervical cancer and cell polarity. *Oncogene* 27, 7018–7030.

Vinokurova, S., and von Knebel Doeberitz, M. (2011). Differential methylation of the HPV 16 upstream regulatory region during epithelial differentiation and neoplastic transformation. *PLoS ONE* 6, e24451. doi:10.1371/journal.pone.0024451

Wiklund, L., Sokolowski, M., Carlsson, A., Rush, M., and Schwartz, S. (2002). Inhibition of translation by UAUUUAU and UAUUUUUAU motifs of the AU-rich RNA instability element in the HPV-1 late 3′ untranslated region. *J. Biol. Chem.* 277, 40462–40471.

Wilson, R., Fehrmann, F., and Laimins, L. A. (2005). Role of the E1–E4 protein in the differentiation-dependent life cycle of human papillomavirus type 31. *J. Virol.* 79, 6732–6740.

Wilson, R., Ryan, G. B., Knight, G. L., Laimins, L. A., and Roberts, S. (2007). The full-length E1E4 protein of human papillomavirus type 18 modulates differentiation-dependent viral DNA amplification and late gene expression. *Virology* 362, 453–460.

Wooldridge, T. R., and Laimins, L. A. (2008). Regulation of human papillomavirus type 31 gene expression during the differentiation-dependent lifecycle through histone modifications and transcription factor binding. *Virology* 374, 371–380.

Wu, S.-Y., Lee, A.-Y., Hou, S. Y., Kemper, J. K., Erdjument-Bromage, H., Tempst, P., and Chiang, C.-M. (2012). Brd4 links chromatin targeting to HPV transcriptional silencing. *Genes Dev.* 20, 2383–2396.

Zhao, X., Fay, J., Lambkin, H., and Schwartz, S. (2007). Identification of a 17-nucleotide splicing enhancer in HPV-16 L1 that counteracts the effect of multiple hnRNP A1-binding splicing silencers. *Virology* 369, 351–363.

Zhao, X., Rush, M., and Schwartz, S. (2004). Identification of an hnRNP A1-dependent splicing silencer in the human papillomavirus type 16 L1 coding region that prevents premature expression of the late L1 gene. *J. Virol.* 78, 10888–10905.

Zheng, Z. M., and Wang, X. (2011). Regulation of cellular miRNA expression by human papillomaviruses. *Biochim. Biophys. Acta* 1809, 668–677.

Zimmermann, H., Degenkolbe, R., Bernard, H. U., and O'Connor, M. J. (1999). The human papillomavirus type 16 E6 oncoprotein can down-regulate p53 activity by targeting the transcriptional coactivator CBP/p300. *J. Virol.* 73, 6209–6219.

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Biased amplification of human papillomavirus DNA in specimens containing multiple human papillomavirus types by PCR with consensus primers

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Genotyping human papillomavirus (HPV) in clinical specimens is important because each HPV type has different oncogenic potential. Amplification of HPV DNA by PCR with the consensus primers that are derived from the consensus sequences of the *L1* gene has been used widely for the genotyping. As recent studies have shown that the cervical specimens often contain HPV of multiple types, it is necessary to confirm whether the PCR with the consensus primers amplifies multiple types of HPV DNA without bias. We amplified HPV DNA in the test samples by PCR with three commonly used consensus primer pairs (L1C1/L1C2+C2M, MY09/11, and GP5+/6+), and the resultant amplicons were identified by hybridization with type-specific probes on a nylon membrane. L1C1/L1C2+C2M showed a higher sensitivity than the other primers, as defined by the ability to detect HPV DNA, on test samples containing serially diluted one of HPV16, 18, 51, 52, and 58 plasmids. L1C1/L1C2+C2M failed to amplify HPV16 in the mixed test samples containing HPV16, and either 18 or 51. The three consensus primers frequently caused incorrect genotyping in the selected clinical specimens containing HPV16 and one or two of HPV18, 31, 51, 52, and 58. The data indicate that PCR with consensus primers is not suitable for genotyping HPV in specimens containing multiple HPV types, and suggest that the genotyping data obtained by such a method should be carefully interpreted. (*Cancer Sci*, doi: 10.1111/j.1349-7006.2011.01922.x, 2011)

Human papillomavirus (HPV), composed of an icosahedral capsid and a circular double-stranded DNA genome, is classified into more than 100 genotypes based on the nucleotide sequence homology of the *L1* gene encoding the major capsid protein.[1] The HPV types found in lesions of the skin and genital mucosa are grouped as cutaneous and genital HPVs, respectively. Of genital HPVs, 15 types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 73) that have been found in cervical cancer are called high-risk HPVs[2] and the types, such as HPV6 and HPV11, that have been found in benign genital warts are called low-risk HPVs.[3]

For detection and genotyping of HPV DNA in the clinical specimens, such as cervical swabs and Pap smears, a part of the *L1* gene is amplified by PCR then grouped based on the susceptibility to various restriction enzymes, the binding capacity to type-specific probes, or the nucleotide sequences of the amplicons.[4] Several consensus primer pairs have been developed and used as standard primers for PCR-based genotyping of HPV in the clinical specimens. L1C1/L1C2+C2M was developed in 1991,[5] and has been used in more than 10 articles describing HPV prevalence in the Japanese population.[5–16] MY09/11[17] and GP5+/6+[18] were developed in 1989 and 1995, respectively, and have been used in numerous studies worldwide.[19] These primers are derived from the consensus sequences of the *L1* gene and the amplicons contain type-specific sequences. Recently new primers, PGMY09/11[20] and modified GP5+/6+ (MGP),[21] which are composed of several type-specific primers, were developed to improve the accuracy of HPV genotyping. The World Health Organization HPV Laboratory Network, which was founded to improve the quality of laboratory services for effective surveillance and monitoring of HPV vaccination impact, recommends PCR with PGMY09/11 followed by reverse blotting hybridization with type-specific probes, as a standard procedure for HPV genotyping.[22]

Recent studies showed that many HPV-positive women are infected with multiple genotypes.[23,24] Therefore, the methods capable of detecting and genotyping HPV DNA of multiple types in a single clinical specimen are necessary to know the precise prevalence of HPV types and the impact of HPV vaccines. As PCR does not always amplify different DNA fragments with equal efficiency, we examined whether PCR with consensus primers can amplify HPV DNA of multiple genotypes in a single sample without bias. We found that PCR with consensus primers sometimes results in mistyping.

Materials and Methods

Plasmids. The pUC plasmid containing the complete genome of HPV16, 18, 31, 51, or 52, or the *L1* gene of HPV58 was used. Purified plasmids were quantified with the NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). The copy numbers of HPV genomes were calculated from the concentration of plasmid expressed as molarities and Avogadro's number.

Clinical specimens. Cervical exfoliated cells were collected from outpatients who visited the NTT Medical Center Tokyo, with their informed consent. The study design and sample collection were approved by the institutional review board. One case of normal cytology, two cases of cervical intraepithelial neoplasia (CIN) grade 1, two cases of CIN2, two cases of CIN3, and one case of unknown cytology were selected for this study. DNA was purified using the QIAamp DNA blood kit (Qiagen, Hilden, Germany).

Polymerase chain reaction. Table 1 shows the sequences of primers used in this study: three consensus primer pairs, L1C1/L1C2+C2M,[5] MY09/11,[17] and GP5+/6+;[18] and two mixtures of type-specific primers, PGMY09/11[20] and modified GP5+/6+ (MGP).[21] Polymerase chain reaction amplification was done in a 50 μL reaction mixture containing 1× PCR buffer II (Life Technologies, Carlsbad, CA, USA), 1.25 units AmpliTaq Gold DNA polymerase (Life Technologies), and 50 ng cellular DNA extracted from human HaCaT cells. The 5′-end of either the forward or reverse primer was biotinylated. The concentrations of MgCl2, dNTPs, and primers, and the reaction temperature were adjusted to those used in the original article

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Table 1. Nucleotide sequences of primers used in this study

	Primer set	Forward (5′–3′)	Reverse (5′–3′)
Consensus primers	L1C1/L1C2+C2M	CGTAAACGTTTTCCCTATTTTTTT	TACCCTAAATACTCTGTATTG
			TACCCTAAATACCCTATATTG
	MY09/11	GCMCAGGGWCATAAYAATGG	CGTCCMARRGGAWACTGATC
	GP5+/6+	TTTGTTACTGTGGTAGATACTAC	GAAAAATAAACTGTAAATCATATTC
Multiple primers	PGMY09/11	GCACAGGGACATAACAATGG	CGTCCCAAAGGAAACTGATC
		GCGCAGGGCCACAATAATGG	CGACCTAAAGGAAACTGATC
		GCACAGGGACATAATAATGG	CGTCCAAAAGGAAACTGATC
		GCCCAGGGCCACAACAATGG	GCCAAGGGGAAACTGATC
		GCTCAGGGTTTAAACAATGG	CGTCCCAAAGGATACTGATC
			CGTCCAAGGGGATACTGATC
			CGACCTAAAGGGAATTGATC
			CGACCTAGTGGAAATTGATC
			CGACCAAGGGGATATTGATC
			GCCCAACGGAAACTGATC
			CGACCCAAGGGAAACTGGTC
			CGTCCTAAAGGAAACTGGTC
			GCGACCCAATGCAAATTGGT
	MGP	ACGTTGGATGTTTGTTACTGTGGTGGATACTAC	ACGTTGGATGGAAAAATAAACTGTAAATCATATTCCT
		ACGTTGGATGTTTGTTACCGTTGTTGATACTAC	ACGTTGGATGGAAAAATAAATTGTAAATCATACTC
		ACGTTGGATGTTTGTTACTAAGGTAGATACCACTC	ACGTTGGATGGAAATATAAATTGTAAATCAAATTC
		ACGTTGGATGTTTGTTACTGTTGTGGATACAAC	ACGTTGGATGGAAAAATAAACTGTAAATCATATTC
		ACGTTGGATGTTTGTTACTATGGTAGATACCACAC	ACGTTGGATGGAAAAATAAACTGCAAATCATATTC

MGP, modified GP5+/6+.

Fig. 1. Amplicons produced from the human papillomavirus (HPV)16*L1* gene by PCR with primers tested in this study. Both ends of amplicons are indicated by the nucleotide (nt) numbers of the HPV16*L1* gene. MGP, modified GP5+/6+.

describing the method.[5,17,18,20,21] Figure 1 shows the location of primers on the HPV16L1 ORF and the size of amplicons.

Reverse blotting hybridization. Reverse blotting hybridization was done as described previously.[22] Briefly, 15 μL denatured amplicons, of which the 5′-ends were labeled with biotin, were allowed to hybridize with the type-specific probes immobilized on a nylon membrane using the Miniblotter MN45 (Immunetics, Cambridge, MA, USA). The hybridized amplicon was detected using streptavidin–HRP (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and ECL detection reagents (GE Healthcare Bio-Sciences). The chemiluminescence was detected with the Light-Capture AE-6972 (ATTO, Tokyo, Japan). The intensities of dots were quantified by ImageJ software (National Institutes of Health, Bethesda, MD, USA). The specific density was calculated by the subtraction of the background from the integrated density. Samples showing the specific densities of more than 1000 intensity units were defined as positives.

The nucleotide sequences of type-specific probes for MY09/11, GP5+/6+, PGMY09/11, and MGP primers were described previously.[25,26] Type-specific probes for L1C1/L1C2+C2M primers were newly designed in this study. The nucleotide sequences were as follows: HPV16, 5′-GTTATTGTTAGGTTTTTTAA; HPV18, 5′-CCACCACCTGCAGGAACCCT; HPV31, 5′-AGGATTGTCAGATTTAGGTA; HPV51, 5′-TAGCAGCACGCGTTGAGGTT; HPV52, 5′-ACCATTACCACTACTGGTGT; and HPV58, 5′-TATTGTTATTGGGACTTTTG.

Real-time PCR. Copy numbers of HPV DNA in a clinical sample were determined by real-time PCR using type-specific primers and SYBR-green dye. A reaction mixture (20 μL) containing 2 μL sample, 10 μL Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan), 0.4 μL ROX reference dye, and 0.9 μM each primer was subjected to PCR with the Applied Biosystems 7900HT (Life Technologies). The reaction was done in triplicate. The copy number was calculated with the standard curve obtained by using serially diluted HPV plasmids. The nucleotide sequences of type-specific primers are as follows: HPV16 forward, 5′-CAGAACCATATGGCGACAGC and reverse, 5′-GTACATTTTCACCAACAGCA; HPV18 forward, 5′-GATTATTTACAAATGTCTGCA and reverse, 5′-GCACAGTGTCACCCATAGTA; HPV31 forward, 5′-GATTATCTTAAAATGGTTGCT and reverse, 5′-GGACCGATTCACCAACCGTG; HPV51 forward, 5′-AGCTATGGATTTCGCTGCCC and reverse, 5′-AGCAAAGATTTGCTCCCTGC; HPV52 forward, 5′-GATTATTTGCAAATGGCTAGC and reverse, 5′-GGCACAGGGTCACCTAAGGTA; HPV58 forward, 5′-AGTGAACCTTATGGGGATAG and reverse, 5′-AAAGGTCATCCGGGA-

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CAGCC. Polymerase chain reaction with these primer sets amplified target HPV DNA without non-specific reaction. For example, PCR with the primers for HPV16 and a test sample containing HPV16 produced a single DNA fragment that formed a single peak in the dissociation curve.

Results

Amplification of HPV DNA in a test sample containing a single HPV genotype. Figure 2 shows the results of reverse blotting hybridization of the amplicons obtained by PCR from test samples (50 μL) containing 6, 60, 600, or 6000 copies of the plasmid having genomic DNA of HPV16, 18, 51, 52, and 58, all prevalent types among Japanese women, and 50 ng sheared human DNA. Three consensus primer pairs (L1C1/L1C2+C2M, MY09/11, and GP5+/6+) and two sets of mixed multiple primers (PGMY09/11 and MGP) were used for PCR. The biotinylated amplicons were allowed to hybridize with the type-specific probes immobilized on a nylon membrane and the biotin on the membrane was detected by streptavidin labeled with peroxidase.

Polymerase chain reaction with L1C1/L1C2+C2M produced detectable amplicons of HPV16, 18, 51, 52, and 58 from the samples containing 60, 6, 6, 60, and 60 copies of HPV DNA, respectively. Polymerase chain reaction with MY09/11 produced detectable amplicons of HPV16, 18, and 58 from the samples containing 600 copies of HPV DNA but did not produce detectable amplicons from the samples containing 6000 copies of HPV51 and 52. Polymerase chain reaction with GP5+/6+ produced detectable amplicons of HPV16, 18, 52, and 58 from the samples containing 60, 60, 6000, and 6000 copies, respectively, but failed to produce detectable amplicons from the sample containing 6000 copies of HPV51. Thus, PCR with L1C1/L1C2+C2M amplified HPV in the test samples more efficiently than the other PCR with the consensus primers.

Polymerase chain reaction with PGMY09/11 produced detectable amplicons of HPV16, 18, 51, 52, and 58 from the samples containing 6, 6, 60, 60, and 60 copies of HPV DNA, respectively. The PCR with MGP produced detectable amplicons of HPV16, 18, 51, 52, and 58 from samples containing 6, 60, 60, 6, and 600 copies of HPV DNA, respectively.

Amplification of HPV DNA in a mixed test sample containing HPV16, and either HPV18, 51, 52, or 58. Figure 3 shows the results of reverse blotting hybridization of the amplicons obtained by PCR from test samples containing 6000 copies of HPV16 and 6, 60, 600, and 6000 copies of either HPV18, 51, 52, or 58 and 50 ng sheared human DNA.

Polymerase chain reaction with L1C1/L1C2+C2M failed to amplify HPV16 DNA from the samples containing 6000 copies of HPV18 or 51 and the level of HPV16 amplicons was greatly reduced in the presence of 600 copies of HPV18 or 51. However, the amplification of HPV18 and 51 DNA was not influenced by the presence of 6000 copies of HPV16 DNA (Figs 2,3). The PCR with L1C1/L1C2+C2M amplified HPV16 DNA in the presence of HPV52 or 58 DNA. The data clearly indicate that amplification of HPV16 DNA by PCR with L1C1/L1C2+C2M was significantly interfered with by the presence of HPV18 or 51 DNA.

Polymerase chain reaction with MY09/11, GP5+/6+, PGMY09/11, or MGP amplified HPV16 DNA in the presence of HPV18, 51, 52, or 58 DNA but the level of HPV16 amplicons was reduced by co-existence of 6000 copies of HPV18 DNA. The PCR with GP5+/6+, PGMY09/11, or MGP produced reduced levels of HPV18, 52, or 58 amplicons in the co-existence of 6000 copies of HPV16 DNA (Figs 2,3).

Amplification of HPV DNA in clinical specimens containing two or three HPV genotypes. Table 2 shows the detection and genotyping of HPVs in clinical specimens using PCR with consensus primers. Eight clinical samples in which two or three HPV types had been detected by PCR with PGMY09/11 or MGP were selected, and the copy numbers of the detected HPV DNA in the samples were measured by real-time PCR using type-specific primers. Then the HPV in the samples was examined by PCR with L1C1/L1C2+C2M, MY09/11, or GP5+/6+. The HPV types detected with consensus primers are listed in decreasing order of amplicon levels.

In no. 165 containing HPV16 (16 000 copies), 18 (5200), and 31 (3100), HPV18 was detected by PCR with L1C1/L1C2+C2M but neither HPV16, which was three times more abundant than HPV18, nor 31 were detected. All three HPVs were detected by PCR with MY09/11. HPV16 and 18 were

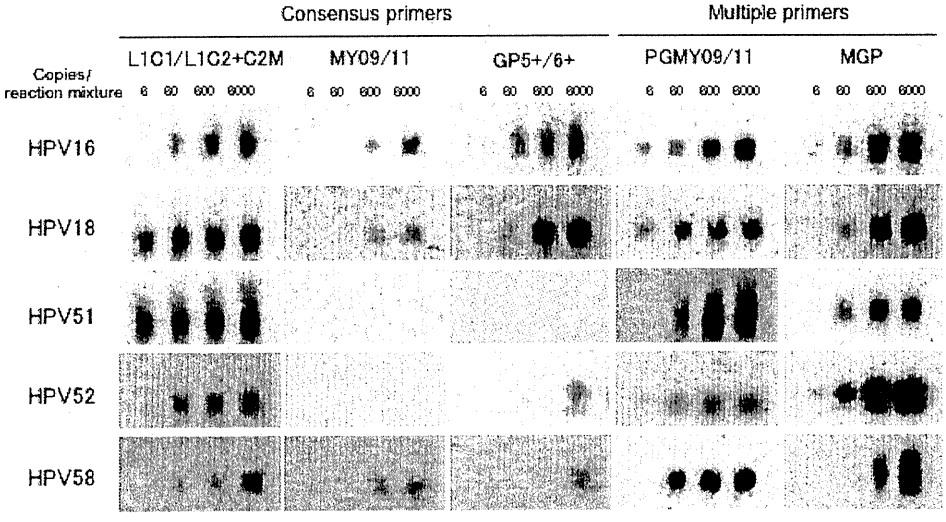

Fig. 2. Amplification of human papillomavirus (HPV) DNA in test samples containing HPV DNA of single genotype. The HPV DNA in the test sample was amplified by PCR with primers indicated. The test sample contained 6, 60, 600, or 6000 copies of plasmid DNA having HPV genomic DNA of the indicated type. The biotin-labeled amplified DNA fragments were hybridized with type-specific probes fixed on a membrane and reacted with streptavidin–HRP. MGP, modified GP5+/6+.

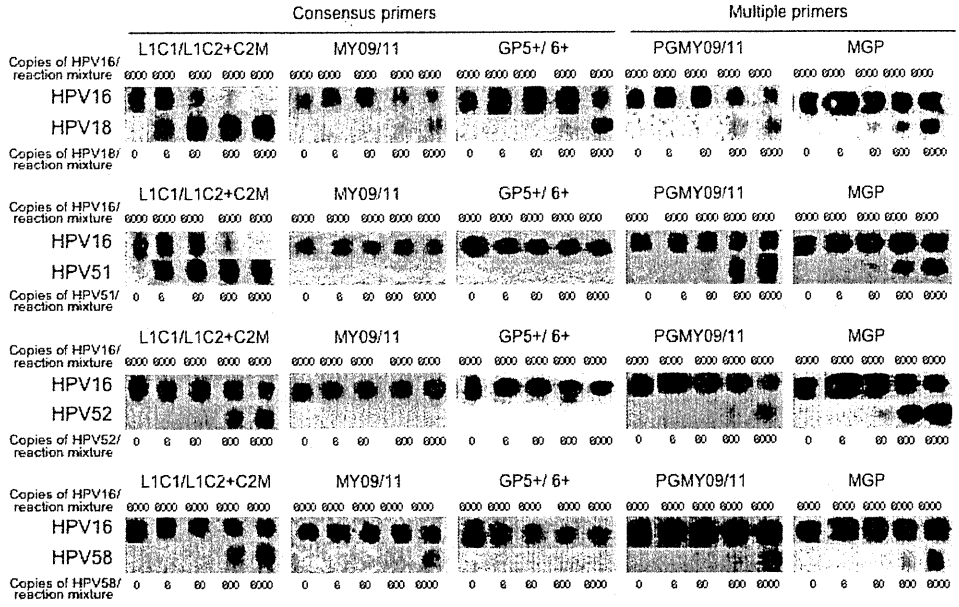

Fig. 3. Amplification of human papillomavirus (HPV) DNA in test samples containing HPV16 DNA and either HPV18, 51, 52, or 58 DNA. HPV DNA in the test sample was amplified by PCR with primers indicated. The test sample contained 6000 copies of HPV16 plasmid together with 6, 60, 600, or 6000 copies of HPV18, 51, 52, or 58 plasmid. The biotin-labeled amplified DNA fragments were hybridized with type-specific probes fixed on a membrane and reacted with streptavidin-horseradish peroxidase. MGP, modified GP5+/6+.

Table 2. Genotyping of clinical samples containing multiple types of human papillomavirus (HPV)

Sample no. (cytology)	Genotypes: copy numbers†	Consensus primers		
		L1C1/ L1C2+C2M	MY09/11	GP5+/6+
165 (CIN2)	16:16 000	18	18‡	16
	18:5200		16	18
	31:3100		31	
258 (CIN3)	52:1700	52	16	16
	16:1300	16	58	
	58:180	58		
352 (normal)	16:200		16	16
	52:25			
402 (CIN2)	52:7300	51	52	16
	51:120	52	16	
	16:110			
993 (CIN1)	16:5600	16	16	16
	52:3300	52		
996 (CIN1)	16:47 000	16	16	16
	52:25 000	52		
1061 (unknown)	18:4300	18	18	18
	16:290		16	16
1245 (CIN3)	16:78 000	58	58	16
	58:78 000	16	16	

†Genotypes detected using PGMY09/11 and modified GP5+/6+ primers. Copy numbers were determined by real-time PCR and expressed as copy numbers subjected to PCR for genotyping. ‡The HPV types detected with consensus primers are listed in decreasing order of amplicon levels.

detected by PCR with GP5+/6+ but HPV31, the least component, was not detected by PCR with GP5+/6+.

In no. 258 containing HPV52 (1700), 16 (1300), and 58 (180), all three HPVs were detected by PCR with L1C1/L1C2+C2M. HPV16 and 58 were detected by PCR with MY09/11 but HPV52, the most abundant type, was not detected. Only HPV16 was detected by PCR with GP5+/6+.

In no. 352 containing HPV16 (200) and 52 (25), neither HPVs were detected by PCR with L1C1/L1C2+C2M. Only HPV16 was detected by PCR with MY09/11 and PCR with GP5+/6+.

In no. 402 containing HPV52 (7300), 51 (120), and 16 (110), HPV16 was not detected by PCR with L1C1/L1C2+C2M. HPV16 and 52, but not HPV51, were detected by PCR with MY09/11. Only HPV16 was detected by PCR with GP5+/6+.

In no. 933 containing HPV16 (5600) and 52 (3300), and in no. 996 containing HPV16 (47 000) and 52 (25 000), HPV52 was not detected by PCR with MY09/11 and GP5+/6+.

In no. 1061 containing HPV18 (4300) and 16 (290), HPV16 was not detected by PCR with L1C1/L2C2+C2M.

In no. 1245 containing HPV16 (78 000) and 58 (78 000), HPV58 was not detected by PCR with GP5+/6+. Thus, amplification of HPV DNA in the clinical specimens containing multiple HPV genotypes by PCR with consensus primers was biased and sometimes caused misjudgment in typing.

Discussion

We evaluated HPV consensus primers for PCR amplification of HPV DNA in specimens containing multiple HPV types and concluded that PCR with consensus primers is not suitable for simultaneous amplification of multiple types of HPV DNA. The low sensitivity of HPV51 and 52 detection by PCR with MY09/11 and GP5+/6+ is consistent with previous reports.[20,21,27] Polymerase chain reaction with L1C1/L1C2+C2M, which amplified HPV DNA tested more efficiently than PCR with MY09/11 or GP5+/6+ (Fig. 2), failed to amplify HPV16 DNA when HPV18 or 51 DNA coexisted in the samples (Fig. 3, Table 2).

Polymerase chain reaction with L1C1/L1C2+C2M amplifies HPV18 and 51 DNA very efficiently, as shown in Figure 2. The resultant abundant HPV18 and 51 amplicons might inhibit reactions amplifying the other HPV types in the samples. Similarly, it is reported that interference in PCR was observed even

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between two closely related plant virus isolates that have identical binding sites for consensus primers, although the mechanism of the interference is not fully explained.[28]

As PCR with L1C1/L1C2+C2M has been used widely,[5–16] the biased amplification may have caused mistyping in the previous numerous studies of genotyping of clinical specimens. Previous studies using L1C1/L1C2+C2M showed that the rate of multiple infections among HPV-positive Japanese women was 10–20%.[15,16] In other countries, studies using PGMY09/11 showed that 30–45% of HPV-positive women were infected with multiple genotypes.[20,29–31] It is possible that reevaluation of HPV prevalence in Japanese women by using mixtures of type-specific primers, such as PGMY09/11 and MGP, increases the rate of multiple infections. The data of this study suggest that the genotyping data obtained by PCR with consensus primers should be carefully interpreted.

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Disclosure Statement

The authors have no conflict of interest to declare.

References

1 Howley PM, Lowy DR. Papillomaviruses and their replication. In: Lnipe DM, Howley PM, eds. *Fields Virology*, Vol. 2, 4th edn. Philadelphia, PA: Lippincott, Williams and Wilkins, 2001; 2197–229.
2 Munoz N, Bosch FX, Castellsague X *et al.* Against which human papillomavirus types shall we vaccinate and screen? *Int J Cancer* 2004; **111**: 278–85.
3 Lacey CJ, Lowndes CM, Shah KV. Chapter 4: Burden and management of non-cancerous HPV-related conditions: HPV-6/11 disease. *Vaccine* 2006; **24**: S3/35–41.
4 Molijn A, Kleter B, Quint W, van Doorn LJ. Molecular diagnosis of human papillomavirus (HPV) infections. *J Clin Virol* 2005; **32**: S43–51.
5 Yoshikawa H, Kawana T, Kitagawa K, Mizuno M, Yoshikura H, Iwamoto A. Detection and typing of multiple genital human papillomaviruses by DNA amplification with consensus primers. *Jpn J Cancer Res* 1991; **82**: 524–31.
6 Iwasaka T, Matsuo N, Yokoyama M, Uchiyama K, Fukuda H, Sugimori H. Prospective follow-up of Japanese women with cervical intraepithelial neoplasia and various human papillomavirus types. *Int J Gynecol Obstet* 1998; **62**: 269–77.
7 Yoshikawa H, Nagata C, Noda K *et al.* Human papillomavirus infection and other risk factors for cervical intraepithelial neoplasia in Japan. *Br J Cancer* 1999; **80**: 621–4.
8 Machama T, Asato T, Kanazawa K. Prevalence of HPV infection in cervical cytology-normal women in Okinawa, Japan, as determined by a polymerase chain reaction. *Int J Gynaecol Obstet* 2000; **69**: 175–6.
9 Maehama T, Asato T, Kanazawa K. Prevalence of human papillomavirus in cervical swabs in the Okinawa Islands, Japan. *Arch Gynecol Obstet* 2002; **267**: 64–6.
10 Masumoto N, Fujii T, Ishikawa M *et al.* Papanicolaou tests and molecular analyses using new fluid-based specimen collection technology in 3000 Japanese women. *Br J Cancer* 2003; **88**: 1883–8.
11 Asato T, Machama T, Nagai Y, Kanazawa K, Uezato H, Kariya K. A large case-control study of cervical cancer risk associated with human papillomavirus infection in Japan, by nucleotide sequencing-based genotyping. *J Infect Dis* 2004; **189**: 1829–32.
12 Masumoto N, Fujii T, Ishikawa M *et al.* Dominant human papillomavirus 16 infection in cervical neoplasia in young Japanese women; study of 881 outpatients. *Gynecol Oncol* 2004; **94**: 509–14.
13 Machama T. Epidemiological study in Okinawa, Japan, of human papillomavirus infection of the uterine cervix. *Infect Dis Obstet Gynecol* 2005; **13**: 77–80.
14 Takakuwa K, Mitsui T, Iwashita M *et al.* Studies on the prevalence of human papillomavirus in pregnant women in Japan. *J Perinat Med* 2006; **34**: 77–9.
15 Onuki M, Matsumoto K, Satoh T *et al.* Human papillomavirus infections among Japanese women: age-related prevalence and type-specific risk for cervical cancer. *Cancer Sci* 2009; **100**: 1312–6.
16 Matsumoto K, Oki A, Furuta R *et al.* Predicting the progression of cervical precursor lesions by human papillomavirus genotyping: a prospective cohort study. *Int J Cancer* 2010; doi: 10.1002/ijc.25630.
17 Manos MM, Ting Y, Wright DK, Lewis AJ, Broker TR, Wolinsky SM. The use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. *Cancer Cell* 1989; **7**: 209–14.
18 de Roda Husman AM, Walboomers JM, van den Brule AJ, Meijer CJ, Snijders PJ. The use of general primers GP5 and GP6 elongated at their 3′ ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. *J Gen Virol* 1995; **76**: 1057–62.
19 Clifford GM, Smith JS, Plummer M, Muñoz N, Franceschi S. Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. *Br J Cancer* 2003; **88**: 63–73.
20 Gravitt PE, Peyton CL, Alessi TQ *et al.* Improved amplification of genital human papillomaviruses. *J Clin Microbiol* 2000; **38**: 357–61.
21 Söderlund-Strand A, Carlson J, Dillner J. Modified general primer PCR system for sensitive detection of multiple types of oncogenic human papillomavirus. *J Clin Microbiol* 2009; **47**: 541–6.
22 World Health Organization, Department of Immunization, Vaccines and Biologicals. Human papillomavirus laboratory manual, 1st edn. 2009. Available from URL: http://www.who.int/biologicals/vaccines/hpv/en/index.html.
23 Mendez F, Munoz N, Posso H *et al.* Cervical coinfection with human papillomavirus (HPV) types and possible implications for the prevention of cervical cancer by HPV vaccines. *J Infect Dis* 2005; **192**: 1158–65.
24 Schmitt M, Dondog B, Waterboer T, Pawlita M, Tommasino M, Gheit T. Abundance of multiple high-risk human papillomavirus (HPV) infections found in cervical cells analyzed by use of an ultrasensitive HPV genotyping assay. *J Clin Microbiol* 2010; **48**: 143–9.
25 Gravitt PE, Peyton CL, Apple RJ, Wheeler CM. Genotyping of 27 human papillomavirus types by using L1 consensus PCR products by a single-hybridization, reverse line blot detection method. *J Clin Microbiol* 1998; **36**: 3020–7.
26 Schmitt M, Bravo IG, Snijders PJ, Gissmann L, Pawlita M, Waterboer T. Bead-based multiplex genotyping of human papillomaviruses. *J Clin Microbiol* 2006; **44**: 504–12.
27 Chan PK, Cheung TH, Tam AO *et al.* Biases in human papillomavirus genotype prevalence assessment associated with commonly used consensus primers. *Int J Cancer* 2006; **118**: 243–5.
28 Rosner A, Maslenin L. Interference in PCR: transcription amplifications of mixed PVY isolates. *Plant Prot Sci* 2005; **41**: 125–31.
29 Coutlée F, Gravitt P, Kornegay J *et al.* Use of PGMY primers in L1 consensus PCR improves detection of human papillomavirus DNA in genital samples. *J Clin Microbiol* 2002; **40**: 902–7.
30 Klug SJ, Hukelmann M, Hollwitz B *et al.* Prevalence of human papillomavirus types in women screened by cytology in Germany. *J Med Virol* 2007; **79**: 616–25.
31 van Doorn LJ, Quint W, Kleter B *et al.* Genotyping of human papillomavirus in liquid cytology cervical specimens by the PGMY line blot assay and the SPF(10) line probe assay. *J Clin Microbiol* 2002; **40**: 979–83.

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NOTE

Novel multiplexed genotyping of human papillomavirus using a VeraCode-allele-specific primer extension method

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ABSTRACT

A VeraCode-allele-specific primer extension (ASPE) method was applied to the detection and genotyping of human papillomavirus (HPV)-DNA. Oligonucleotide primers containing HPV-type-specific L1 sequences were annealed to HPV-DNA amplified by PGMY-PCR, followed by ASPE to label the DNA with biotinylated nucleotides. The labeled DNA was captured by VeraCode beads through hybridization, stained with a streptavidin-conjugated fluorophore, and detected by an Illumina BeadXpress® reader. By using this system, 16 clinically important HPV types (HPV6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) were correctly genotyped in a multiplex format. The VeraCode-ASPE genotyping of clinical DNA samples yielded identical results with those obtained by validated PGMY-reverse blot hybridization assay, providing a new platform for high-throughput genotyping required for HPV epidemiological surveys.

Key words allele-specific primer extension, genotyping, human papillomavirus, multiplex assay.

Human papillomaviruses (HPV) are recognized as the causative agents of cervical cancer, its precursor lesions, 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 potentials (2). A previous large-scale case–control study revealed 15 high-risk 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, with HPV16 the predominant high-risk type worldwide (3). In contrast, low-risk HPV types, including HPV6 and 11, are associated almost exclusively with benign lesions. Due to the lack of a cell culture system to isolate HPV from clinical samples, detection of HPV-DNA is the only reliable means for diagnosis of HPV infection. HPV genotyping is of particular importance for understanding the natural history of HPV infection and management of cervical cancers. In addition, with the worldwide introduction of HPV vaccines that target the two prominent high-risk types, HPV16 and 18, there is a growing demand for reliable and practical HPV genotyping to monitor HPV prevalence and vaccine efficacy at both individual and population levels.

Various molecular techniques have been developed for detection of HPV-DNA, most of which rely on amplification of HPV-DNA by PCR. The PCR of HPV-DNA generally utilizes degenerate/consensus primer systems, such as MY09/11 (4), PGMY09/11 (5), GP5+/6+ (6), or SPF (7), all of which are designed to amplify the L1 region of the HPV genome. For HPV genotyping, PCR is followed

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List of Abbreviations: ASPE, allele-specific primer extension; HPV, human papillomavirus; MFI, median fluorescence intensity; RBH, reverse blot hybridization; RLB, reverse line blot; SNP, single nucleotide polymorphism; WHO, World Health Organization.

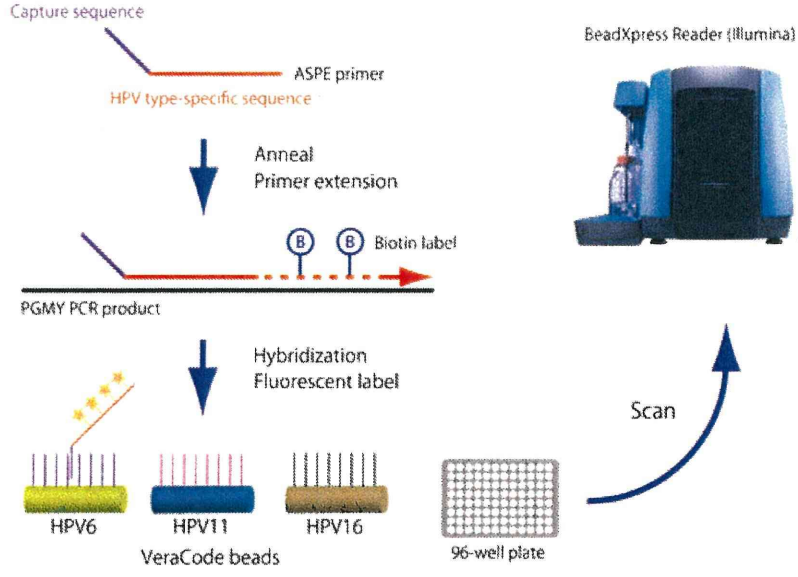

Fig. 1. Outline of VeraCode-ASPE HPV genotyping. HPV-type-specific ASPE primers are annealed to PGMY-PCR products, followed by primer extension by DNA polymerase to label DNA with biotin-14-dCTP. The biotin-labeled DNA is hybridized with VeraCode beads via a capture sequence at the 5′ end of the ASPE primer, and labeled with a streptavidin-fluorophore conjugate to allow detection of fluorescent signals by a BeadXpress® reader in a 96-well plate format. Each VeraCode bead contains digitally assigned barcodes so that the BeadXpress® reader can identify HPV-type-specific signals from each VeraCode-bead, enabling HPV genotyping with digital read-out. The BeadXpress® reader photograph is used with the permission of Illumina Inc. (San Diego, CA, USA).

by sequence analysis, restriction fragment length polymorphism analysis, or hybridization with type-specific oligonucleotide probes by a membrane-based RLB assay. Of the various HPV genotyping assays, the RLB assay has the advantage of being able to detect multiple HPV-type infections with greater sensitivity. Several RLB assays combined with different PCR schemes have been established and used for HPV research and diagnostic purposes (8–10). However, the RLB assays are relatively laborious, are limited to a maximum of about 40 samples per assay, and depend on visual read-out of the hybridization signal. To overcome these drawbacks, HPV genotyping using Luminex® suspension array technology has been developed (11–14). The Luminex®-based genotyping coupled with GP5+/6+ PCR allowed sensitive and specific genotyping of 27 mucosal HPV types in a 96-well plate format with a digital read-out (13). Moreover, a modified version of GP5+/6+ PCR was successfully introduced into the Luminex®-based assay, and showed improved sensitivity (15).

A VeraCode-ASPE method was first developed for the detection of SNP in the human genome (16) and has been applied to multiplex SNP genotyping on the Illumina BeadXpress® platform (17, 18). The ASPE primer is composed of two distinct regions: the 5′ region that contains the capture sequence, which is used in a subsequent hybridization reaction, and the 3′ region that contains the genomic target region with a SNP nucleotide at the extreme 3′ end. For SNP genotyping, the ASPE primer that matches the SNP nucleotide to the genome is extended by the primer extension reaction and is thus labeled with biotinylated nucleotides. After the primer extension, the products are mixed with VeraCode beads, so that the capture sequence on the primer hybridizes to its complementary sequence attached to the VeraCode beads. Labeling is then carried out with a streptavidin-fluorophore conjugate, followed by scanning and detection of the fluorescent signal using an Illumina BeadXpress® reader (Illumina Inc., San Diego, CA, USA).

In this work, the VeraCode-ASPE method on the Illumina BeadXpress® platform was evaluated for its suitability as a method to detect and genotype HPV-DNA (Fig. 1). The HPV-DNA amplified by PGMY-PCR was selected as a target for the VeraCode-ASPE genotyping, as PGMY-PCR has been validated as a sensitive and specific means for HPV-DNA amplification (19, 20). HPV-type-specific ASPE primers were designed to target the PCR amplicons of 16 HPV types (HPV6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) in the 3′ region (Table 1), and with type-specific capture sequences in the 5′ region. The T_m values of the HPV-type-specific sequences, the lengths of which ranged from 19 to 28 bases, were adjusted to be between 54°C and 66°C using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) thus allowing similar annealing profiles.

HPV-DNA, which was provided by the HPV laboratory network in the WHO as a quality-assured authentic panel for validation of HPV genotyping, was used to assess the sensitivity and specificity of the VeraCode-ASPE HPV genotyping. Fifty copies of HPV16- and 18-DNA and 500 copies of the other 14 HPV-type DNAs from the panel were subjected to PGMY-PCR with AmpliTaq Gold® polymerase (Applied Biosystems, Foster City, CA,

Table 1. HPV-type-specific sequences in ASPE primers for VeraCode-ASPE genotyping

Type	DNA sequence (5′–3′)	Tm (°C)
HPV6	CATCCGTAACTACATCTTCCA	55.3
HPV11	GCATCTGTGTCTAAATCTGCTAC	55.9
HPV16	AGTACCTACGACATGGGGAG	56.6
HPV18	CCAGGTACAGGAGACTGTGTA	55.3
HPV31	TGCAATTGCAAACAGTGATAC	57.3
HPV33	CTTTATGCACACAAGTAACTAGTGA	55.7
HPV35	TGTTCTGCTGTGTCTTCTAGTGA	57.8
HPV39	CCAACTTTACATTATCTACCTCTATAGA	55.3
HPV45	CACAAAATCCTGTGCCAAGT	58.6
HPV51	ACTGCTGCGGTTTCCCCAA	65.6
HPV52	GGAATACCTTCGTCATGGC	57.9
HPV56	TGATGCACGAAAAATTAATCAG	57.9
HPV58	TGACATTATGCACTGAAGTAACTAAG	57.0
HPV59	AAAGAATATGCCAGACATGTG	55.3
HPV66	AGTTAATGTGCTTTTAGCTGC	54.3
HPV68	GCTGTACCAAATATTTATGATCCTAA	57.1

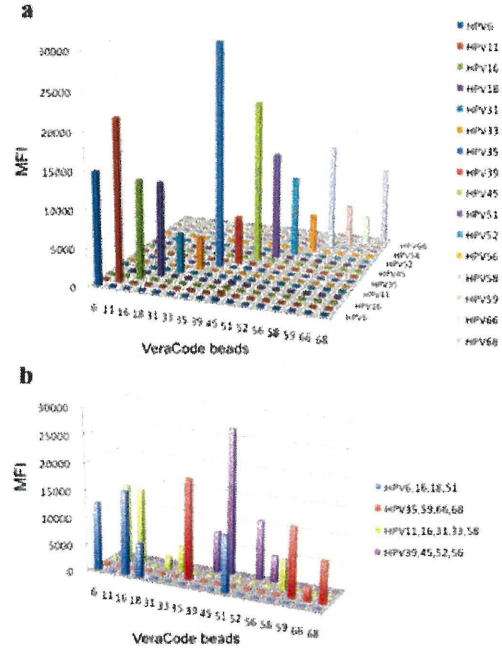

Fig. 2. VeraCode-ASPE genotyping of the WHO HPV-DNA proficiency panel. (a) PGMY-PCR products derived from the panel DNA containing each single-type HPV-DNA were subjected to the VeraCode-ASPE reaction and analyzed by the BeadXpress® reader. The signals from each VeraCode bead are shown as the MFI. The VeraCode bead number indicates the HPV type number that is assigned to each bead. (b) PGMY-PCR products derived from the panel DNA containing multiple-type HPV-DNA were subjected to the VeraCode-ASPE reaction and analyzed by the BeadXpress® reader.

USA) as described (21). One-third of the PCR products was treated with 2 U shrimp alkaline phosphatase and 5 U exonuclease I at 37°C for 45 min, followed by the ASPE reaction in a mixture containing 1× PCR buffer II (Roche, Indianapolis, IN, USA), 2.5 mM $MgCl_2$, 5 μM of each dATP, dGTP and dTTP, 7.5 μM biotin-14-dCTP, 0.05 μM of each ASPE primer, 0.5 U AmpliTaq Gold® polymerase, with denaturation at 95°C for 10 min followed by 50 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 45 sec. The reaction products were then incubated with the VeraCode bead mixture for 1 hr at 45°C in a VeraCode-bead plate, followed by staining with streptavidin-Alexa-647 in a buffer consisting of 3× standard saline citrate (SSC) and 0.1% Tween 20 for 15 min at room temperature. The VeraCode-bead plate was subjected to scanning by the BeadXpress® reader, and the read-out was expressed as the MFI obtained from each HPV type-assigned bead. As shown in Figure 2a, the 16 types of HPV-DNA were specifically detected with signals from their corresponding VeraCode beads. Signal values from non-target HPV-DNAs were as low as those from DNA-negative samples, and were classified as background noises. Furthermore, when the panel DNA containing a mixture of HPV-DNA was analyzed, corresponding signals from included HPV types were correctly detected (Fig. 2b), which indicates that VeraCode-ASPE typing is applicable to the simultaneous detection of multiple HPV-type DNAs.

To test the suitability of this assay for diagnostic purposes, DNA samples prepared from clinical specimens were analyzed by VeraCode-ASPE HPV genotyping. DNA was purified using the QIAamp® DNA blood kit (QIAGEN, Hilden, Germany) from cervical exfoliated cells that had been collected from outpatients with their informed consent for HPV genotyping. The study design was approved by the institutional review board of the NTT Medical Center, Tokyo. DNA samples were previously genotyped by PGMY-reverse blot hybridization (PGMY-RBH) assay, which had been validated as to be sensitive and specific for genotyping of the 16 HPV types in the studies of the WHO HPV-DNA proficiency panel (20). The same PGMY-PCR products derived from these DNA samples were subjected to VeraCode-ASPE HPV genotyping as carried out for the WHO HPV-DNA panel. A positive result was defined as a signal value more than three-fold the average background value for each HPV-type-specific VeraCode bead. Of 50 clinical samples analyzed by the VeraCode-ASPE assay, 20 samples gave HPV-positive results, whereas the remaining 30 samples were judged to be negative. Table 2 shows raw MFI data and typing results of the VeraCode-ASPE assay with 20 positive samples and one negative sample. Overall, the typing results were identical to those obtained by the PGMY-RBH assay, which strongly suggests that the VeraCode-ASPE assay can

Table 2. VeraCode-ASPE genotyping of DNA from clinical specimens

DNA no.	HPV6 HPV45 Typing result	HPV11 HPV51	HPV16 HPV52	HPV18 HPV56	HPV31 HPV58	HPV33 HPV59	HPV35 HPV66	HPV39 HPV68
#1644	46	50	47	102	53	47	53	78
	58	**28537**	50	51	48	71	424	147
	HPV51							
#1646	233	185	209	298	194	207	204	224
	213	320	237	**14999**	243	215	672	262
	HPV56							
#1647	203	237	**14047**	281	219	215	191	240
	217	297	200	224	217	**19118**	647	307
	HPV16, 59							
#1650	221	243	214	296	246	255	221	231
	253	323	**12271**	260	251	229	669	333
	HPV52							
#1652	47	54	51	140	57	46	56	97
	59	140	**24978**	55	56	55	608	173
	HPV52							
#1654	201	212	186	316	206	202	216	258
	821	289	219	191	**14963**	197	743	312
	HPV45, 58							
#1657	389	383	318	434	353	363	334	**1570**
	363	445	**46218**	395	364	346	825	431
	HPV39, 52							
#1661	120	130	128	238	166	136	149	**4599**
	156	221	151	134	123	137	622	255
	HPV39							
#1662	242	221	**45495**	244	186	223	238	270
	229	279	**26066**	209	230	231	518	286
	HPV16, 52							
#1666	204	252	**2654**	301	236	191	225	265
	223	330	226	243	**16990**	225	556	**5996**
	HPV16, 58, 68							
#1668	81	83	**38858**	135	92	84	86	121
	95	148	**10338**	84	94	83	435	178
	HPV16, 52							
#1669	118	156	122	189	**7566**	129	148	151
	143	207	140	131	**30234**	151	449	224
	HPV31, 58							
#1672	209	209	214	281	220	203	170	241
	219	266	216	201	**22802**	218	578	290
	HPV58							
#1676	91	92	96	169	96	87	92	121
	100	**12816**	89	99	96	97	491	190
	HPV51							
#1679	279	274	240	**36487**	238	268	278	270
	257	325	293	232	276	265	519	354
	HPV18							
#1683	241	278	**39325**	312	276	232	246	266
	249	321	251	233	258	226	552	337
	HPV16							
#1684	91	87	104	130	88	87	94	103
	96	145	89	101	86	91	**26638**	160
	HPV66							
#1685	151	129	**6983**	237	150	122	137	174
	150	221	146	145	145	139	559	233
	HPV16							

Continued.

Table 2. Continued

DNA no.	HPV6 HPV45 Typing result	HPV11 HPV51	HPV16 HPV52	HPV18 HPV56	HPV31 HPV58	HPV33 HPV59	HPV35 HPV66	HPV39 HPV68
#1686	196	198	198	289	169	204	235	203
	208	276	202	187	186	**20644**	574	275
	HPV59							
#1691	230	205	230	346	227	234	216	222
	250	275	225	**13139**	226	241	552	317
	HPV56							
#1645	150	138	142	252	151	136	148	183
	147	219	143	148	134	131	659	229
	negative							
DNA(–)	188	205	202	289	195	198	213	211
	217	264	206	220	204	222	457	259
Cut-off	565	615	607	868	584	595	638	634
	650	791	618	659	611	667	1372	778

Signal values above cut-off values are indicated in bold letters.

substitute for the reverse blot hybridization on the same platform of PGMY-PCR.

The principle of the allele-specific primer extension was previously used in tag-array-based HPV genotyping (22, 23); however, the array format of this assay hampers its application to high-throughput HPV genotyping. In contrast, the 96-well plate format of the VeraCode-ASPE method enables HPV genotyping for large amounts of clinical samples. Furthermore, there are a total of 144 different sets of VeraCode beads, and thus it is possible to include more HPV types in the VeraCode-ASPE genotyping format. In conclusion, the VeraCode-ASPE genotyping is a powerful new tool for the high-throughput HPV genotyping that will be required for large-scale surveillance of HPV-type distribution at the population level in the near future.

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DISCLOSURE

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REFERENCES

1. Zur Hausen H. (2002) Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer* **2**: 342–50.
2. De Villiers E.M., Fauquet C., Broker T.R., Bernard H.U., Zur Hausen H. (2004) Classification of papillomaviruses. *Virology* **324**: 17–27.
3. Munoz N., Bosch F.X., De Sanjose S., Herrero R., Castellsague X., Shah K.V., Snijders P.J., Meijer C.J. (2003) Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med* **348**: 518–27.
4. Coutlee F., Hankins C., Lapointe N. (1997) Comparison between vaginal tampon and cervicovaginal lavage specimen collection for detection of human papillomavirus DNA by the polymerase chain reaction. The Canadian Women's HIV Study Group. *J Med Virol* **51**: 42–7.
5. Gravitt P.E., Peyton C.L., Alessi T.Q., Wheeler C.M., Coutlee F., Hildesheim A., Schiffman M.H., Scott D.R., Apple R.J. (2000) Improved amplification of genital human papillomaviruses. *J Clin Microbiol* **38**: 357–61.
6. De Roda Husman A.M., Walboomers J.M., Van Den Brule A.J., Meijer C.J., Snijders P.J. (1995) The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. *J Gen Virol* **76**(Pt 4): 1057–62.
7. Kleter B., Van Doorn L.J., Ter Schegget J., Schrauwen L., Van Krimpen, K., Burger M., Ter Harmsel B., Quint W. (1998) Novel short-fragment PCR assay for highly sensitive broad-spectrum detection of anogenital human papillomaviruses. *Am J Pathol* **153**: 1731–39.
8. Van Den Brule A.J., Pol R., Fransen-Daalmeijer N., Schouls L.M., Meijer, C.J., Snijders P.J. (2002) GP5+/6+ PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes. *J Clin Microbiol* **40**: 779–87.
9. Gravitt P.E., Peyton C.L., Apple R.J., Wheeler, C.M. (1998) Genotyping of 27 human papillomavirus types by using L1 consensus PCR products by a single-hybridization, reverse line blot detection method. *J Clin Microbiol* **36**: 3020–27.

10. Kleter B., Van Doorn, L.J., Schrauwen L., Molijn A., Sastrowijoto S., Ter Schegget J., Lindeman J., Ter Harmsel B., Burger M., Quint W. (1999) Development and clinical evaluation of a highly sensitive PCR-reverse hybridization line probe assay for detection and identification of anogenital human papillomavirus. *J Clin Microbiol* **37**: 2508–17.
11. Schmitt M., Bravo I.G., Snijders P.J., Gissmann L., Pawlita M., Waterboer T. (2006) Bead-based multiplex genotyping of human papillomaviruses. *J Clin Microbiol* **44**: 504–12.
12. Oh Y., Bae S.M., Kim Y.W., Choi H.S., Nam G.H., Han S.J., Park C.H., Cho Y., Han B.D., Ahn W.S. (2007) Polymerase chain reaction-based fluorescent Luminex assay to detect the presence of human papillomavirus types. *Cancer Sci* **98**: 549–54.
13. Schmitt M., Dondog B., Waterboer T., Pawlita M. (2008) Homogeneous amplification of genital human alpha papillomaviruses by PCR using novel broad-spectrum GP5+ and GP6+ primers. *J Clin Microbiol* **46**: 1050–59.
14. Nazarenko I., Kobayashi L., Giles J., Fishman C., Chen G., Lorincz A. (2008) A novel method of HPV genotyping using Hybrid Capture sample preparation method combined with GP5+/6+ PCR and multiplex detection on Luminex XMAP. *J Virol Methods* **154**: 76–81.
15. Soderlund-Strand A., Carlson J., Dillner J. (2009) Modified general primer PCR system for sensitive detection of multiple types of oncogenic human papillomavirus. *J Clin Microbiol* **47**: 541–46.
16. Lin C.H., Yeakley J.M., Mcdaniel T.K., Shen R. (2009) Medium- to high-throughput SNP genotyping using VeraCode microbeads. *Methods Mol Biol* **496**: 129–42.
17. Tokarska M., Marshall T., Kowalczyk R., Wojcik J.M., Pertoldi C., Kristensen T.N., Loeschcke V., Gregersen V.R., Bendixen C. (2009) Effectiveness of microsatellite and SNP markers for parentage and identity analysis in species with low genetic diversity: the case of European bison. *Heredity* **103**: 326–32.
18. Mefford H.C., Cooper G.M., Zerr T., Smith J.D., Baker C., Shafer N., Thorland E.C., Skinner C., Schwartz C.E., Nickerson D.A., Eichler E.E. (2009) A method for rapid, targeted CNV genotyping identifies rare variants associated with neurocognitive disease. *Genome Res* **19**: 1579–85.
19. Mori S., Nakao S., Kukimoto I., Kusumoto-Matsuo R., Kondo K., Kanda T. (2011) Biased amplification of human papillomavirus DNA in specimens containing multiple human papillomavirus types by PCR with consensus primers. *Cancer Sci* **102**: 1223–7.
20. Estrade C., Menoud P.A., Nardelli-Haefliger D., Sahli R. (2011) Validation of a low-cost HPV genotyping assay baced on PGMY-PCR and reverse blotting hybridization with reusable membranes. *J Clin Microbiol* **49**: 3474–81.
21. Unger E.R., Dillner J., Zhou T. (2009) *Human Papillomavirus Laboratory Manual*, 1st edition. Geneva, Switzerland: WHO.
22. Kaller M., Hultin E., Zheng B., Gharizadeh B., Wallin K.L., Lundeberg J., Ahmadian A. (2005) Tag-array based HPV genotyping by competitive hybridization and extension. *J Virol Methods* **129**: 102–12.
23. Gheit T., Landi S., Gemignani F., Snijders P.J., Vaccarella S., Franceschi S., Canzian F., Tommasino M. (2006) Development of a sensitive and specific assay combining multiplex PCR and DNA microarray primer extension to detect high-risk mucosal human papillomavirus types. *J Clin Microbiol* **44**: 2025–31.

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Rsf-1 (HBXAP) expression is associated with advanced stage and lymph node metastasis in ovarian clear cell carcinoma

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Summary

Ovarian clear cell carcinoma (CCC) is a unique type of ovarian cancer characterized by distinct clinicopathological and molecular features. CCC is considered to be a highly malignant disease because it is resistant to conventional chemotherapy, and, when presented at advanced stages, has a dismal overall survival. Identifying and characterizing biomarkers associated with its malignant behavior is fundamental toward elucidating the mechanisms underlying its aggressive phenotype. In this study, we performed immunohistochemical analysis on 89 CCCs to assess their expression of *Rsf-1 (HBXAP)*, a chromatin remodeling gene frequently amplified and overexpressed in several types of human cancer. We found that 73 (82%) of 89 CCCs expressed Rsf-1 and most importantly, there was a statistically significant correlation between Rsf-1 immunostaining intensity and two disease parameters: advanced stage ($p= 0.008$) and status of retroperitoneal lymph node metastasis ($p= 0.023$). However, there was no correlation between Rsf-1 expression and patient age, peritoneal tumor dissemination, or overall survival. In conclusion, a higher expression level of Rsf-1 is associated with advanced clinical stage and lymph node metastasis in CCC. Our data suggest that Rsf-1 participates in tumor progression in CCC, and indicates that the contribution of Rsf-1 to disease aggressiveness deserves further study.

Introduction

Ovarian clear cell carcinoma (CCC) represents less than 10% of ovarian cancers in the United States, but occurs more frequently in Asian women (1,2). Multivariate analysis on a large series of CCC shows that women with CCC present at a younger age and at earlier clinical stages as compared to high-grade (conventional) serous carcinoma, the most common and lethal type of ovarian cancer (1). Approximately 50% of CCCs present as stage I diseases (3,4) and, despite being diagnosed at an early stage, are generally considered to be

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highly malignant (5). Morphological and molecular studies have demonstrated that many CCCs develop in a stepwise fashion from endometriosis through atypical endometriosis to overt CCC (6–10). In fact, CCC is the most common ovarian carcinoma associated with endometriosis. There has been increased enthusiasm for identifying markers that are predictive of the clinical outcome in CCC patients. This is because CCC typically presents with stage I or II disease, and prognostic markers could have an impact on clinical decision making in the management of CCC patients, such as administration of adjuvant chemotherapy. For example, IGF2BP3 (IMP3) expression has been reported to be an independent marker of reduced disease-specific survival in CCC, but not in high-grade serous or endometrioid carcinomas of the ovary (11). Similarly, enhanced expression of annexin A4 in CCC and its association with chemoresistance to carboplatin have been recently reported (12).

To further identify markers that are associated with poor prognosis in CCC and to explore the molecular mechanisms that account for the aggressive behavior of CCC, we determined the correlation between immunoreactivity of Rsf-1, also known as HBXAP, and clinical outcome in primary CCCs. We focused on Rsf-1 (HBXAP) because the encoded protein participates in chromatin remodeling, and this gene has been identified as an amplified gene with a tumor-promoting potential in several types of neoplastic diseases including ovarian high-grade serous carcinoma (13,14,15). Our analysis showed that higher expression levels of Rsf-1 (HBXAP) were associated advanced stage disease and retroperitoneal lymph node metastasis. The current study provides new evidence of the biological significance of Rsf-1 expression in CCC.

Materials and methods

Tissue samples

Formalin-fixed and paraffin-embedded CCC tissues were obtained from the Department of Pathology at the University of Tokyo Hospital. A total of 89 cases of primary CCCs were retrieved from the archives, and hematoxylin and eosin (H&E) stained slides were reviewed to confirm the diagnosis based on the most recent criteria of the World Health Organization. The CCC tissues were arranged in tissue microarrays (Beecher Instruments, Silver Spring, MD) with duplicate 2 mm tissue cores obtained from the tumor area in each CCC. The collection of clinical specimens was in compliance of guideline of tissue procurement at the University of Tokyo Hospital.

Clinical information of patients with ovarian clear cell carcinoma

We reviewed the medical records from all 89 CCC patients; data obtained included demographics, age at the time of diagnosis, preoperative diagnosis, clinical stage, and survival time after treatment. None of the patients underwent preoperative chemotherapy or radiotherapy. The correlations of Rsf-1 expression with the following clinical variables were evaluated: age, stage of carcinoma (stage I/II *vs.* stage III/IV), peritoneal dissemination, retroperitoneal lymph node metastasis, and death rate. Stage of carcinoma was assessable in 67 cases in which the appropriate staging procedures were performed; the remaining 22 cases were not included in the staging analysis due either to incomplete surgical procedures or to missing data. Staging was in accordance with the standards of the International Federation of Gynecology and Obstetrics (FIGO). Comprehensive evaluation of peritoneal dissemination that included microscopic examination of the omentum, peritoneal wall and mesentery soft tissues was performed in 79 cases. Retroperitoneal lymph node dissection was performed in 70 cases. Follow-up information included overall survival and cancer-related death. The follow-up interval was calculated from the date of surgery to the date of