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**Invited Review**

# Genetic Variations of Human Papillomavirus Type 16: Implications for Cervical Carcinogenesis

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**CONTENTS:**



**SUMMARY:** Human papillomaviruses (HPVs) are the causative agent of cervical cancer, and among approximately 15 high-risk genotypes, HPV16 accounts for more than half the cases of cervical cancer worldwide. Recent progress in determining HPV genomic sequences from clinical samples has revealed a wide variety in HPV16 genome sequences, and has allowed for comprehensive classification of intratype HPV16 variants. These consist of four variant lineages containing nucleotide variations in 1.0%–10.0% of the complete viral genome sequence. Epidemiological data suggest that the non-European–Asian lineages of HPV16 entail a higher risk of progression to invasive cervical cancer than the European–Asian lineage. Deep sequencing analysis has recently demonstrated that HPV16 genome sequences are highly homogeneous in individual clinical specimens compared with those of RNA viruses. However, an extremely sensitive PCR method, differential DNA denaturation PCR, has detected hypermutations from C to T or G to A in the *E2* gene and the long control region of the HPV16 genome, which suggests the involvement of cellular apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) proteins in this hypermutation. The quasispecies status of the HPV16 genome in the infected cervix may affect the development of cervical cancer and warrants further investigation.

## 1. HPV infection and cervical cancer

Human papillomaviruses (HPVs) are a group of nonenveloped DNA viruses, with an approximately 8,000-bp, circular double-stranded DNA genome, encapsidated within the viral capsid. The viral genome is typically composed of eight open reading frames (E1, E2, E4, E5, E6, E7, L1, and L2) and a noncoding long control region (LCR) (1). The early genes *E1*, *E2*, *E4*, *E5*, *E6*, and *E7* are required for replication, transcription, and maintenance of the viral genome in infected cells, whereas the late genes *L1* and *L2* encode the major and minor capsid proteins, respectively. The LCR contains the viral origin of replication and the early promoter, which drives the expression of the *E6* and *E7* genes. Their gene products target cellular tumor suppressor proteins, including TP53 and RB, for functional inactivation.

Persistent infection with a subset of HPV genotypes, referred to as ''high-risk'' types, is causally linked to the development of cervical, vulvar, vaginal, anal, and penile cancers, as well as a proportion of head and neck cancers (2). Epidemiologically, at least 15 HPV genotypes (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) are recognized as high-risk types (3), among which HPV16 accounts for more than half the cases of cervical cancer worldwide (4). Figure 1 schematically illustrates the natural history of HPV-induced cervical carcinogenesis. High-risk HPV infects the basal cells in the cervical epithelium through microlesions and establishes its genome as an extrachromosomal episome. HPV genomic amplification is closely linked to the epithelial differentiation program and often induces hyperplasia of the epithelial cells, which manifests clinically as cervical intraepithelial neoplasia grade 1 (CIN1). However, the majority (~90%) of incidental infections are cleared by the host immune system within a few years (5,6), and only a small proportion persist and progress further to CIN grade 2 or 3 (CIN2/3), which are the precursors of invasive cervical cancer (ICC). The final steps in cervical carcinogenesis, triggering the development of ICC, are not fully understood,

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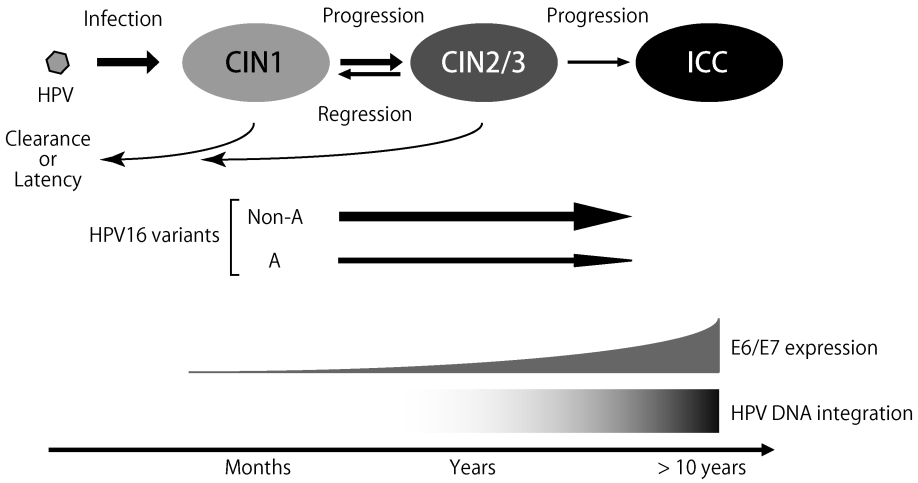


Fig. 1. Natural history of cervical carcinogenesis induced by persistent HPV16 infection. Progression to CIN2/3 and ICC is accompanied by the enhanced expression of viral oncoproteins E6 and E7. HPV DNA is frequently integrated into the host genome in ICC. Epidemiological data suggest that non-lineage A variants tend to progress to CIN2/3 or ICC more readily than lineage A variants.

but it has been frequently observed that HPV DNA is integrated into the genomes of cancerous cells, which also express high levels of *E6/E7*. Because *E2* encodes a viral transcriptional repressor for the early promoter, which is responsible for *E6/E7* expression, the disruption of *E2* by viral integration enhances *E6/E7* expression and strongly favors the development of ICC (2).

Although high-risk HPV DNA is detected in nearly all cases of cervical cancer, HPV infection alone is not sufficient to drive full carcinogenesis. The development of ICC usually requires persistent infection with a high-risk HPV for more than 10 years, during which time the host genome accumulates multiple mutations in the cellular oncogenes or tumor suppressor genes, ultimately resulting in the generation of ICC.

## 2. Genetic diversity of HPV16

HPVs display an astonishing variety in their genomic sequences, and those with more than 10% nucleotide differences in their *L1* sequences are defined as distinct genotypes (7). So far, traditional PCR-based cloning and recent next-generation sequencing (NGS) techniques have led to the identification of the complete genome sequences of more than 170 HPV genotypes (8), and the total number of genotypes continues to increase. In contrast, HPV genomes with less than 10% differences in their *L1* sequences are recognized as subtypes or intratype variants (9). This constitutes an additional level of HPV genetic complexity. Because HPV infects only the human mucosa or epithelium and depends on highly reliable host DNA synthesis for the replication of its genome, the genetic diversity of HPVs is considered to have been generated during the co-evolution of humans and HPV during the long history of the host–virus interaction (10).

Because of the predominance of HPV16 in cases of cervical cancer, the risk of cervical cancer associated with HPV16 infection has been studied at the level of its intratype variants, and many lines of evidence attribute a higher risk of progression to ICC to some distinct lineages of HPV16 variants (9). The HPV16 variants are classified into different lineages and sublineages, defined as containing 1.0%–10.0% and 0.5%–1.0% nucleotide variations in the complete viral genome sequence, respectively (11). These include lineage A (previously called European–Asian), which includes sublineages A1, A2, A3 (European, EUR), and A4 (Asian, As); lineage B (African 1, AFR1), consisting of sublineages B1 and B2; lineage C (African 2, AFR2); and lineage D (North American/Asian–American, NA/AA), consisting of sublineages D1 (NA), D2 (AA2), and D3 (AA1). Figure 2 shows an example of the phylogenetic relationships between different HPV16 isolates based on complete viral genome sequences, which are consistent with the lineage assignments based on a unique combination of single-nucleotide polymorphisms (SNPs), and illustrates the vast genetic diversity among the HPV16 variants distributed in nature.

An increased risk of developing CIN2/3 and cervical cancer has been consistently associated with the non-lineage A variants of HPV16 in several studies (Fig. 1) (12–16). In particular, lineage D is reported to be preferentially associated with an increased risk of developing CIN3 or worse (CIN3+) (17–20). Lineage D is also more frequently detected in cervical adenocarcinoma than in squamous cell carcinoma (19,20), which may suggest that this variant lineage displays different biological behaviors in different histological cell types in the cervical epithelium. A recent, worldwide case-control study demonstrated that HPV16 variant lineages are differentially distributed in patients with cervical cancer according to country and region (21), and that the contribution of individual variant lineages to the development of cervical cancer may also differ in different countries and regions. This further complicates the general understanding of the association between specific HPV16 variant lineages and an increased risk of cervical cancer.

In investigating the mechanisms underlying the differential associations between CIN/ICC and HPV16 variant lineages, in vitro cell culture experiments have shown that the LCR and *E6* from some variant lineages confer greater oncogenicity than those from other line-

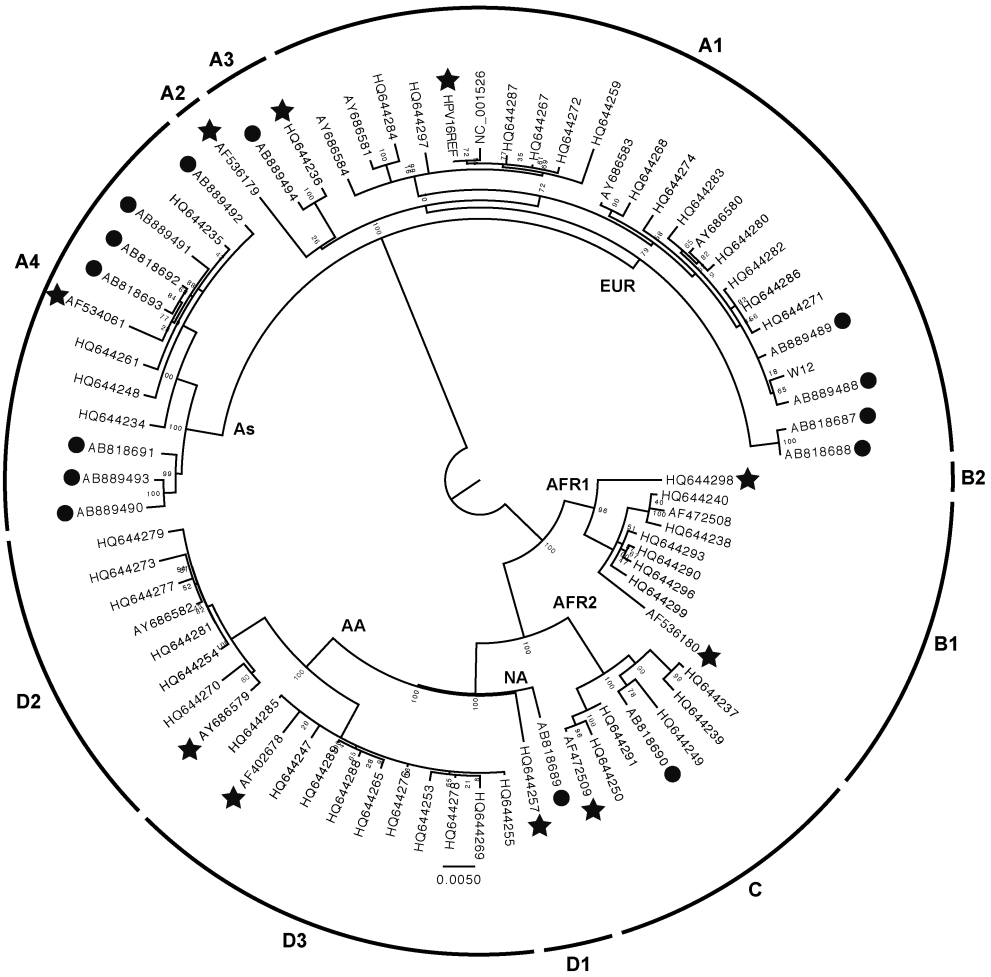


Fig. 2. Phylogenetic tree of complete HPV16 genome sequences. Sixty-two sequences previously reported (18) and 14 sequences determined from Japanese women (marked with closed circles) were aligned using MAFFT. A maximum likelihood phylogenetic tree was constructed using RA × ML, with 1,000-fold bootstrapping. The scale represents a branch length of 0.005, which indicates a 0.5% difference between the nucleotide sequences at the beginning and the end of the branch. The number at each branch node represents the bootstrapping value. Reference sequences of each sublineage are marked with closed stars. HPV16REF, K02718; W12, AF125673.

ages (22–24). Interestingly, the tumorigenic role of *E6* in the D lineage (D2/D3) was recapitulated in a three-dimensional culture model of immortal human keratinocytes in the context of the complete viral genome (25). Another example of the lineage-specific association of *E6* variants with the development of ICC was reported for an A4-sublineage-specific *E6* variant that results in an amino acid change at residue 25 of the E6 protein, from aspartic acid to glutamic acid (D25E), in Chinese (26) and Japanese (27) populations, although this relationship awaits further validation.

Non-lineage-specific SNPs that appear independently of the lineages may also be associated with the risk of cervical cancer. Among the HPV16 non-lineage-specific SNPs, the specific SNP, T350G, has been shown to be associated with an increased risk of cervical cancer. T350G is located in *E6* and causes an amino acid change at residue 83 of the E6 protein, from leucine to valine (L83V) (28–30). However, the association has not been consistently observed in other studies (16,21), which compromises the implication of this particular SNP in cervical carcinogenesis.

Because a growing number of complete HPV genomic sequences are becoming available, the genetic basis of HPV carcinogenicity can be assessed at the nucleotide level by applying a sequence imputation technique, using HPV genomic sequences obtained from clinical samples. This is analogous to a genome-wide association study to identify host SNPs that confer disease susceptibility. Such a viral genome-wide association study was conducted for HPV16 to explore the potential risk of cervical cancer associated with either viral-lineage-specific or -non-specific SNPs (18). In that study, a specific sublineage of lineage D (D3) was shown to have an elevated propensity in causing cancerous lesions. However, because the majority of imputed SNPs correlate strongly with one another as a result of the high lineage fixation in the evolution of the HPV16 genomes, it is difficult to pinpoint the independent causative SNPs that increase the risk of cervical cancer.

## 3. Quasispecies

The concept of viral quasispecies was originally proposed for RNA viruses and refers to viral populations that consist of a variety of genetically different

viruses, rather than a single virus with the same genomic sequence (31). Viral quasispecies primarily result from the use of low-fidelity viral RNA polymerases for genomic replication and play crucial roles in various biological aspects of the RNA viruses. For example, the capacity of RNA viruses to change their cell tropism or host range or to overcome internal and external selective constraints, such as the host immune responses and antiviral agents, depends on the presence of variants in the viral populations in infected cells or tissues. Although the genomic sequences of DNA viruses are relatively stable compared with those of RNA viruses, quasispecies of DNA viruses have also been observed in individual clinical specimens. In the case of HPVs, co-infection with different genotypes is not uncommon in women with CIN, and even in infections with the same genotype, a mixed infection with intratype variants is sometimes observed. These intratype HPV quasispecies have two types of origins: co-infection with multiple variants and mutagenesis arising from a single variant.

The former type of intratype HPV quasispecies can occur during sequential or simultaneous infection with different variants. Supporting this notion, a previous study analyzed the changes in HPV16 variants in cervical swabs and demonstrated that some samples contained multiple HPV16 variants in a single clinical specimen (32), although the detection rate of multiple HPV16 variants in individual women was relatively low (four of 47 women; 8.5%). In that study, of 35 women showing consecutive HPV16-positive results, 32 (91.4%) had the same variant, whereas a change in variant was observed in three (8.6%) women. Another study that analyzed the HPV16 variant status in paired-enrollment and follow-up cervical specimens showed that changes in the HPV16 variants occurred in four of 86 women (4.7%) (33). Among a cohort of human immunodeficiency virus (HIV)-infected adults, eight of 59 women (13.6%) showed discordant HPV16 variants in sequentially collected anal and cervical samples (34). These data suggest that natural infections can result from continuous exposure to infection with different HPV16 variants.

To explain the second type of intratype HPV quasispecies, it is possible that, as in RNA viruses, the HPV genomes maintained in basal epithelial cells gradually accumulate mutations and/or insertions/deletions during persistent infection. This eventually results in a quasispecies status, carrying a variety of minor genetic variations in the viral genome. However, given that the evolutionary rate of nucleotide substitutions in the HPV genome is estimated to be extremely low, approximately $2 \times 10^{-8}$ per site per year (35), because of the high fidelity of the host DNA replication machinery, similar levels of genetic variations observed in the RNA viruses cannot be expected in the HPV genome. Likewise, changes in an HPV16 variant lineage caused by spontaneous mutagenesis, which require at least approximately 40 nucleotide substitutions in the viral genome, are unlikely to occur in individual persistent infections. However, the acquisition of non-lineage-specific SNPs that may facilitate the progression to cervical cancer may occur, which justifies further investigation of such SNPs.

To comprehensively explore the quasispecies status of the HPV16 genome in individual clinical specimens, the mutational landscape in the full-length HPV16 genome was investigated with NGS technology (36). In that study, whole HPV16 genomes were amplified with one-step, long-range PCR from HPV16-positive clinical samples of either CIN1 or ICC, and analyzed by deep sequencing. No co-infections with different variant lineages were detected in the seven samples analyzed, four of which showed highly static viral genomic sequences, with no nucleotide substitutions at a frequency of more than 0.5% anywhere in the viral genome. In contrast, the remaining three samples contained nucleotide substitutions in *E6*, *E1*, or the non-coding region between *E5* and *L2*, with frequencies from 0.60% to 5.42% of the total read depths per nucleotide position. The observed mutation frequencies in the HPV16 genome are considerably lower than those observed for other viruses, such as HIV, hepatitis C virus, and hepatitis B virus (31), indicating that the HPV16 genome is stably maintained during persistent infection. The clinical significance of HPV16 quasispecies, whether related to disease progression or regression, remains to be investigated.

## 4. Hypermutation

An alternative approach was applied to clinical samples to detect low-frequency mutations in the HPV genome (37). Differential DNA denaturation PCR (3D-PCR) uses lower melting temperatures to denature the target DNA than those used in conventional PCR, which favors the selective amplification of A/T-rich DNA and results in the enrichment of A/T-rich mutated DNA in the final PCR product. Using 3D-PCR, hypermutated sequences of the HPV1a and HPV16 genomes were detected in plantar warts and precancerous cervical biopsies, respectively. This hypermutation was observed in the LCR of the HPV1a and HPV16 genomes and was biased for G to A or C to T base change, suggesting the involvement of the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) proteins in generating the hypermutation.

The APOBEC proteins are a family of cellular enzymes that catalyze the deamination of cytidine (C) to uracil (U) on single-stranded DNA/RNA. They function as antiviral factors in the host innate immune system, disrupting the viral genomic information by introducing hypermutation (38). In humans, the APOBEC family has 11 members, including activation-induced cytidine deaminase (AID) and APOBEC1, 2, 3A, 3B, 3C, 3DE, 3F, 3G, 3H, and 4 (39,40). It has been proposed that the APOBEC3 (A3) subfamily is involved in carcinogenesis (41,42). In particular, A3A and A3B are suggested to play an important role in the accumulation of extensive consecutive mutations in multiple human cancers, including cancers of the bladder, cervix, lung, head and neck, and breast (43,44). Intriguingly, A3A and A3B expression levels are upregulated in low- and high-grade cervical lesions compared with normal tissue, and HPV oncoproteins E6/E7 are responsible for this upregulation (45,46), which implies an involvement of A3s in HPV-induced cervical carcinogenesis. It was also recently demonstrated that A3B is upregulated in breast cancer tissues, where high-risk HPV DNA is

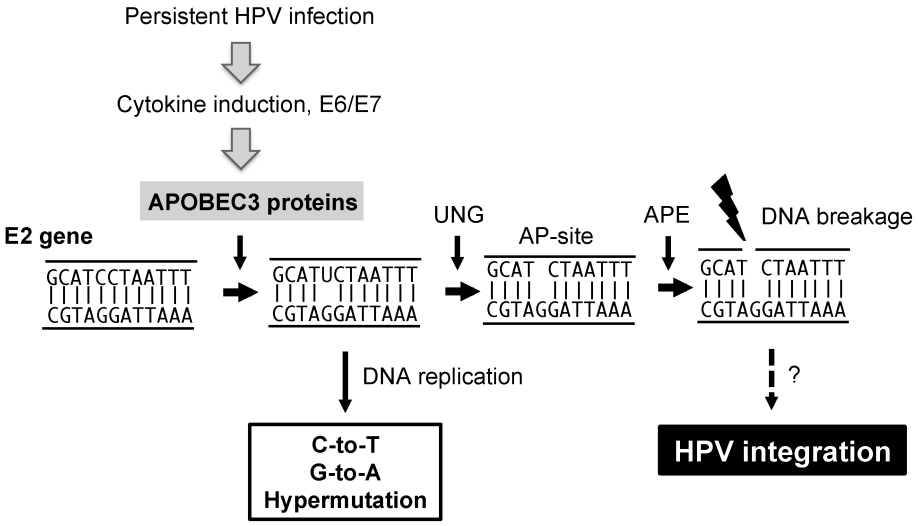


Fig. 3. Model of hypermutation in the HPV16 genome. HPV infection induces expression of cytokines or E6/E7 in cervical keratinocytes, which then leads to the upregulation of APOBEC3 (A3) proteins. The A3s catalyze the deamination of cytidine in the *E2* gene to uracil, which can be converted to thymine and guanine on the opposite DNA strand during DNA replication. In contrast, during the repair process, uracil is removed by uracil-DNA N-glycosylase (UNG), resulting in an apurinic/apyrimidinic (AP) site, and further processed by apurinic/apyrimidinic endonuclease (APE) to induce a DNA strand break, which may facilitate HPV integration into the host genome.

detected (47), suggesting a potential link between HPV infection and A3 induction in breast carcinogenesis. In head and neck cancer, persistent infection with high-risk HPVs has also been reported to promote A3B-mediated mutagenesis in the *PIK3CA* gene (48), which is also frequently mutated in cervical cancer.

An in vitro study using cultured cervical keratinocytes as the model system to analyze persistent HPV infection also supported the primary role of A3s in inducing hypermutation into the HPV16 genome (49). That study demonstrated that when cervical keratinocyte W12 cells (derived from HPV16-positive CIN1 lesions and maintain the HPV16 genome as episomes) are treated with interferon-$\beta$, several members of the A3 subfamily are induced, with concomitant introduction of C-to-T hypermutation in *E2* in the HPV16 genome. This interferon-$\beta$-induced hypermutation is blocked by the transfection of small interfering RNAs against A3G, strongly suggesting the involvement of A3G in the hypermutation of the HPV genome. Similar C-to-T hypermutation in the *E2* sequence of the HPV16 genome has also been detected in clinical specimens, including CIN1 and CIN3 lesions, with 3D-PCR (Kukimoto and Muramatsu, J Med Viol, in press). This indicates that A/T hypermutation in the HPV16 genome is a truly physiological phenomenon, occurring in natural infections.

The physiological relevance of HPV16 hypermutation in the viral life cycle and cervical carcinogenesis has yet to be determined, because hypermutation in *E2* and the LCR in clinical samples seems to be extremely rare in total viral populations, insofar as deep sequencing analysis was unable to detect hypermutated HPV16 sequences at frequencies of more than 0.5% (36). One intriguing possibility is that hypermutation is involved in the integration of HPV into the host genome, a critical event in the generation of cervical cancer, because *E2* is a ''hot spot'' for HPV integration and is frequently disrupted by integration (50). In this context, it is noteworthy that U bases in the cellular immunoglobulin genes generated by AID are processed to double-stranded DNA breaks through the cellular repair pathway. The resulting DNA ends are used for subsequent events, such as class switch recombination, gene conversion, and chromosomal translocation (38). Therefore, it is possible that the generation of U bases in *E2* by A3s also leads to DNA strand breaks, thereby facilitating viral integration (Fig. 3). Because the expression of A3A and A3B also induces double-stranded DNA breaks and genomic instability in the host genome (51–53), DNA strand breaks in both the viral and host genomic DNAs may synergistically increase the chance of viral DNA integration. However, these working hypotheses await further substantiation with in vitro experiments.

## 5. Future perspectives

Although our understanding of the genetic diversity and biological relevance of the HPV16 variants has been greatly extended in this decade, several important questions remain to be answered. First, well-designed large-scale epidemiological studies using complete genome sequences of HPV16 are required to identify the most risky lineage-specific or -non-specific SNPs that lead to the development of cervical cancer. It will be of particular interest to explore how the quasispecies status of the HPV16 genome in clinical specimens, including changes in the viral SNPs and hypermutation in *E2* and the LCR, contributes to cervical carcinogenesis. Therefore, identification of the key changes in the HPV16 genome and clarification of their role in cervical carcinogenesis are important research area and are likely to provide novel strategies for the diagnosis and clinical management of HPV16-infected lesions in the future. Finally, although prophylactic vaccines targeting HPV16 are expected to be effective against all HPV16 variant line-

ages, careful monitoring of the changes in the distributions of HPV16 variants may be necessary to fully assess the effectiveness of these vaccines in the post-vaccination era.

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**Conflict of interest** None to declare.

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