Epigenetics and Human Health

Walter Doerfler
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Epigenetics of Infectious Diseases

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Epigenetics of Infectious Diseases

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Preface

This book describes epigenetic mechanisms in a variety of human pathogens. The list includes viruses, bacteria, and protists. Two chapters deal with the modifications of the host epigenome induced by bacterial and protist infections. We trust that the eleven chapters in this volume will arouse the interest of researchers in epigenetics, virology, microbiology, and infection biology in general, both in molecular biology and in molecular medicine.

An incentive to arrange this volume has been the consideration that the enormous success of epigenetics in higher eukaryotes and its relevance for human health has somehow overshadowed microbial epigenetics. However, epigenetic mechanisms play crucial roles in the lifestyles of viruses, bacteria, protists, and fungi. Such mechanisms are diverse but have an outcome in common: the generation of nongenetic diversity. In pathogens, phenotypic heterogeneity permits the formation of lineages with distinct properties and contributes to the interaction with the eukaryotic host (e.g., evasion of the immune system, division of work, and preadaptation to host-mediated challenges by bet hedging). An equally relevant, emerging notion is that the interaction of microbial pathogens with their hosts can induce changes in the eukaryotic epigenome. The significance of such changes remains poorly understood in many cases. An appealing speculation is that modulation of the host epigenome might provide a memory mechanism that registers the encounter with a pathogen and transmits this information to daughter cells.

Studies critically directed toward epigenetic alterations of virus-infected or virus-transformed cells have so far received limited attention. In contrast, viral systems have been frequently used as models to document the role of DNA methylation in long-term gene silencing in eukaryotes. However, there is increasing evidence to support the notion that virus infections in general can lead to the destabilization of the host cells' epigenetic profiles, probably early on after virus infections. In this volume, the epigenetic consequences for the host genomes upon infections with human papilloma virus, human herpesvirus type 8, human adenovirus type 12, and the human herpesvirus Epstein–Barr virus have been analyzed by research groups active in these fields.

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Chapter 1
DNA Methylation of Human Papillomavirus Genomes During Infection and Cancer Progression

Hans-Ulrich Bernard

Abstract Human papillomaviruses (HPVs) are important pathogens, as they are the cause of all cervical cancers and of subsets of vulval, anal, oral, and penile cancers. The viral genome is an 8 kb double-stranded circular DNA, which can replicate in various types of epithelial cells. HPV DNA shows changes of its methylation profile during the viral life cycle, namely "sporadic" and "polymorphic" DNA methylation associated with low transcription in basal cells of epithelia, and a lack of methylation in suprabasal cells associated with strong transcription. While these epigenetic changes of HPV DNA during the viral life cycle are still poorly understood, it has emerged that during progression of low-grade precursor lesions to malignant carcinomas, the HPV DNA becomes hypermethylated, probably since the viral genome recombines with the chromosomal DNA of the infected host cell. This methylation signal is intensely studied as a candidate biomarker for the diagnosis of HPV-associated lesions that have the potential to progress to cancer.

Keywords Papillomavirus • HPV • Viral life cycle • Cancer progression • Recombination • Insertion

1.1 Introduction

Papillomaviruses are defined by their (1) non-enveloped capsids, (2) circular double-stranded DNA genomes with sizes close to 8000 bp and highly conserved gene organization, (3) host species specificity, (4) tropism for epithelial cells, and (5) transforming rather than lytic effects on the host cells. They cause neoplastic growth of the infected epithelium or can persist in asymptomatic infections.

Papillomavirus genomes have a noncoding region (long control region, LCR) of about 800 bp, which harbors the replication origin, a transcriptional enhancer, and a

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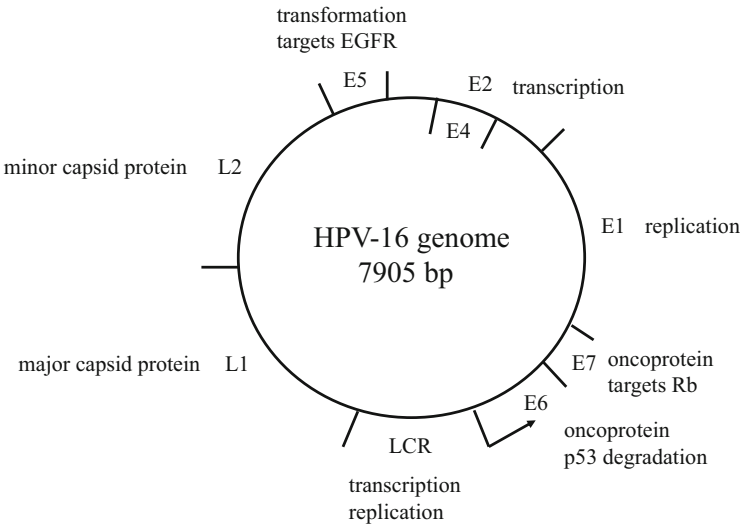

Fig. 1.1 Genome organization of human papillomavirus 16

promoter (Bernard 2013). About half of the genome downstream of the promoter contains the early genes E6, E7, E1, E2, E4, and E5, with the remainder further downstream encoding the late proteins L2 and L1 (Fig. 1.1). For the purpose of this chapter, the following brief summary of the function of these proteins may suffice: E6 and E7 trigger the principal transforming mechanisms, such as interference with p53 and RB cell cycle control (Roman and Munger 2013; vande Pol and Klingelhutz 2013). E1 binds the replication origin and functions as helicase (Bergvall et al. 2013). E2 functions as activating and repressing transcription factor and cooperates with E1 in identification of the replication origin (McBride 2013). And L1 and L2 are the major and the minor capsid proteins (Buck et al. 2013; Wang and Roden 2013).

The papillomavirus life cycle and papillomavirus pathogenesis can be summarized as follows: Papillomaviruses most often infect squamous, i.e., multilayered and differentiating epithelia. In order to establish a stable infection, a papillomavirus particle has to infect the basal layer of such an epithelium, where the circular viral episome persists and replicates. Asymmetric cell divisions of the basal cells lead to suprabasal cells, beginning with the spinous layer. Suprabasal cells normally lack mitotic activity and DNA replication properties. Papillomavirus genomes that are sorted into such cells express E6 and E7 oncoproteins, which target the cellular Rb and p53 proteins, and thereby reestablish an environment of continuing DNA replication and mitoses. The resulting expansion of the suprabasal cell population leads to neoplastic lesions, referred to in the case of skin as “warts.” In cell layers close to the epithelial surface, the virus expresses the capsid proteins, which encapsidate the viral DNA into viral particles that are released upon disintegration of terminally differentiated epithelial surface cells. This life cycle also applies

during nonmalignant infections to those papillomaviruses that are found in cancer, while it becomes distorted during carcinogenesis. All details of carcinogenesis are not yet understood, but early molecular events frequently involve recombination between papillomaviruses and host cell DNA in a genomic arrangement that leads to stimulation of papillomavirus oncogene transcription. For progression to a malignant phenotype, the affected cell has to undergo numerous additional mutations and epigenetic changes of cellular genes (see Mine et al. 2013, and references therein).

For taxonomic purposes, papillomaviruses are referred to as "types," and their names are abbreviated with the letters PV, preceded by one or two letters that define the host, and followed by a number indicating the historic sequence of isolation (Bernard et al. 2010). Among more than 300 papillomavirus types described so far, only the cottontail rabbit papillomavirus (CRPV1) and those human papillomaviruses (HPVs), which are most prevalent in carcinomas of the cervix uteri (HPV16, 18, 31, 33, 35, 45, 52, 58), were addressed by DNA methylation research. No methylation studies have been done with the well-developed cell culture system for bovine papillomavirus-1 (BPV1) or in situ with those HPVs that cause mostly benign lesions such as HPV2 (common warts) or HPV6 (genital warts). These particular PVs are stably maintained as episomes, while the aforementioned PVs can recombine with cellular DNA, what may influence viral DNA methylation.

1.2 History of Papillomavirus Methylation Research

The first records of PV DNA methylation were garnered with CRPV1 (at that time also called Shope papillomavirus), which was shown to have methylated, chromosomally integrated, multicopy viral DNA in rabbit skin tumors (Wettstein and Stevens 1983; Sugarawa et al. 1983). These observations preceded the modern understanding of the regulatory importance of epigenetic alterations. Unfortunately, the CRPV/rabbit system has never been reinvestigated since then. Subsequently, the potential for transcriptional effects of DNA methylation on HPV16 and HPV18 DNA became established in vitro and in cell culture experiments (Thain et al. 1996; Rösl et al. 1993), but these observations were not extended to a search of methylated HPV DNA in situ. Several years later it turned out that HPV DNA methylation is actually a widespread phenomenon, and became observed in a cell line with episomal HPV16 DNA (Kim et al. 2003), in cell lines with integrated HPV16 and HPV18 DNA as well as in carcinomas and their precursor lesions (Badal et al. 2003, 2004; Kalantari et al. 2004). While these studies opened a rich field of investigation, their analytical power was initially limited by the use of methylation sensitive restriction enzymes, and by a focus on small genomic regions, including E2 binding sites. In the last 10 years DNA methylation studies by bisulfite sequencing have targeted larger parts of the genomes or whole genomes of several HPV types in the context of the viral life cycle and carcinogenesis, and these findings will be reviewed in this chapter.

The following questions emerged as the most challenging research objectives:

1. Does DNA methylation affect HPV biology during the normal viral life cycle and are HPVs unique DNA methylation targets, e.g., as a form of cellular defense against foreign DNA?
2. Are there specific regulatory effects of DNA methylation via CpG dinucleotides in E2 binding sites?
3. Do HPVs affect the cellular epigenome?
4. Does DNA methylation differentially affect HPV genomes during progression of asymptomatic infections through precursor lesions to malignant lesions?
5. Are HPV epigenomes or cellular epigenomic properties in HPV infected cells useful biomarkers in the diagnosis of cancer precursor lesions?

1.3 Methylation of HPV DNA During the Normal Life Cycle

In order to understand a potential role of HPV DNA methylation during the normal life cycle, it would be desirable to investigate the DNA first in the viral capsid, then immediately following infection of the basal layer, and further during epithelial differentiation. Unfortunately, HPV research here and elsewhere has always been hampered by the absence of animal models and by difficulties to establish or reproduce cell culture systems. As a consequence, there are presently only two sources of information about the epigenetics of HPVs during the viral life cycle, namely, the epigenetic properties of HPV DNA from patients likely to harbor only episomal DNA and a stable cell line, W12E, that maintains HPV16 DNA episomally.

HPV16, HPV18 and several related high-risk HPV types infect squamous mucosal cells of the female genital tract subclinically, and these infections can progress through cervical cancer precursor lesions (cervical intraepithelial neoplasia I and III, CIN I and CIN III) to invasive cervical cancer. In subclinical infections and CIN I lesions, HPV genomes exist as episomes, while an increasing portion of them recombines during progression. Consequently, it can be assumed that clinical samples obtained from asymptomatic individuals and CIN I patients contain HPVs during the normal viral life cycle. Studies from numerous labs agree that such clinical samples contain "sporadically" methylated HPV genomes, "sporadically" referring to average methylation frequencies per CpG in the range of 5–10%, and a lack of specificity for the CpG target (Kalantari et al. 2004; Turan et al. 2006; Brandsma et al. 2009; Sun et al. 2011; Mirabello et al. 2013). All of these studies addressed CpGs in the LCR and the L1 gene, but some extended the findings throughout the genome. These data constituted methylation analyses of short PCR amplicons. This is a nontrivial limitation, as the sequencing of multiple cloned amplicons from each patient sample found substantial heterogeneity of methylation between HPV genomes from the same sample (Kalantari et al. 2004).

This investigation of clinical samples became complemented by cell culture studies. Among the few cell culture models in papillomavirus research are W12E cells cloned from a CIN lesions of an HPV16-infected patient. The cells grow on a fibroblast feeder layer and morphologically resemble the basal layer of epithelia. Some differentiation can be observed in confluent cultures, and these differentiated cells can be separated from the undifferentiated cells. Lambert and colleagues (Kim et al. 2003) observed a consistent, but only "sporadic" methylation of the HPV16 LCR in undifferentiated cells, similar to patterns observed in situ in cells harvested from asymptomatic or CIN I patients. Most of this methylation was lost upon differentiation of the W12E cells. It is known from studies addressing different aspects of HPV biology that transcription of HPVs becomes activated upon differentiation, and so it is tempting to hypothesize that the observed epigenetic change is the switch between two different transcription states. It should be noted that methylation was only rarely observed at the E2 binding sites overlapping with the E6 promoter, which would activate rather than repress this promoter (see below).

Another study of W12E cells confirmed the methylation of the HPV16 LCR in undifferentiated cells, as well as the ensuing demethylation upon differentiation (Kalantari et al. 2008a). This study also addressed five clonal derivatives of W12E, where all HPV16 genomes had recombined with the cellular DNA. Three of these clones with few HPV16 copies had nearly no methylation of LCR sequences but some methylation of the L1 gene, which is adjacent to the LCR but not transcriptionally affected by its properties. Two clones with numerous HPV16 copies showed strong methylation of the LCR. In contrast to the W12E cells with episomal DNA, differentiation of these five clones with chromosomally integrated viral DNA did not alter HPV16 DNA methylation, neither in the LCR nor in the L1 gene.

1.3.1 In Summary

Studies of clinical samples as well as the W12E line agree that episomal HPV16 DNA is targeted by DNA methylation. DNA methylation is sporadic, i.e., low, and polymorphic both within an individual sample as well as between comparable samples. Differentiated W12E cells contain completely unmethylated HPV16 LCR segments, and such molecules exist in most clinical samples with episomal DNA. It is therefore likely but not mechanistically understood that HPV16 episomes are methylation targets in undifferentiated epithelial cells. This should negatively affect transcriptional activity. Demethylation may release this repression in suprabasal cells and lead to increased transcription, as observed in situ. No evidence suggests a selective recognition of the viral DNA as part of a cellular defense mechanism.

1.4 Regulatory Effects of DNA Methylation via CpG Dinucleotides in E2 Binding Sites

The papillomavirus E2 gene encodes proteins that have the ability to bind the palindromic DNA sequence 5′-ACCGNNNNCGGT-3′, which occurs four times in the LCR of HPV16 and related HPV types. This sequence has two CpG methylation targets, and in vitro studies have shown that E2 proteins cannot bind the methylated target sequences (Thain et al. 1996). As expected, transfection experiments with unmethylated and methylated E2 site reporter genes and E2 factor expression vectors confirmed that methylation dramatically interferes with transcriptional transactivation (Kim et al. 2003).

This straightforward mechanism in vitro is much more complicated in vivo, on the one side due to the expression of different E2 proteins through differential splicing, some being transcriptional activators, some lacking the transcription activation domain, and on the other side due to multiple and opposing functions of E2 binding sites depending on the genomic context. E2 proteins can be (1) activators of transcription when their binding site is remote from a promoter, the binding sites functioning as E2 protein dependent enhancers. Alternatively, they can (2) repress transcription, when they bind target sites at the HPV E6 promoter, in part due to competition between E2 and the promoter factors SP1 and TFIID, whose binding sites overlap with E2 binding sites (Tan et al. 1994), and in part due to E2 complexes with histone modifying proteins (Smith et al. 2014). Lastly, (3) E2 also forms a complex with the replication factor E1 and increases its specificity and affinity to replication initiation sites, and (4) is involved in partitioning of papillomavirus genomes during mitosis (McBride 2013).

HPV methylation studies normally address only the second of these four functions. The reasoning goes as follows: For E2 protein to be expressed, the HPV genome must be continuous from the E6 promoter through the whole E2 gene, as E2 is translated from a polycistronic mRNA containing the E6, E7, E1 and E2 genes. This is the case when HPV genomes are episomal or exist as tandem repeats recombined with chromosomal DNA. In these two cases, the E2 protein serves a repressing feedback loop, binds to the E6 promoter, and decreases its activity. In this scenario, HPVs and their infected cells would have a growth advantage, if the E2 binding sites overlapping with the E6 promoter would be methylated, as E2 protein could not bind and could not lead to repression, increasing the amount of E6 and E7 oncoprotein production. No such advantage of host cells with HPV genomes with methylated E6 promoter sequences exists if no complete E2 transcript (and protein) can be delivered, which is the case when chromosomal recombination led to interruption of the E2 gene, a frequent scenario in cancer (see below).

There is agreement that these scenarios are regularly encountered, but different extents of this mechanism were reported in different studies (Schwarz et al. 1985; Kalantari et al. 2001; Peitsaro et al. 2002; Arias-Pulido et al. 2006; Bhattacharjee and Sengupta 2006; Brandsma et al. 2009; Snellenberg et al. 2012; Chaiwongkot et al. 2013; Mirabello et al. 2013; Bryant et al. 2014). Reasons for disagreement are

technical limits to differentiate between integrated and episomal viral DNA, as, for example, integrated DNA often exists as large concatemers. A role for the E2 protein can be deduced from observations that, typically, the rate of CpG methylation through most of the LCR of HPV16 is by a factor of 2–3 lower than methylation of the four CpGs within the promoter-proximal E2 binding sites, suggesting that clones were selected that have eliminated the negative regulation of the E6 promoter by E2, as this repressor can now not bind anymore to its targets.

1.5 Effects of Papillomaviruses on the Cellular Epigenome

It is well established that extensive epigenomic changes are an intrinsic part of carcinogenesis of all tissues irrespective of their association with papillomaviruses (Sharma et al. 2010), and epigenetic changes contribute to carcinogenesis with a weight similar to that of mutations and aneuplodies. The same applies to cancer of the cervix (Wentzensen et al. 2009; Louvanto et al. 2015; Siegel et al. 2015), and those neoplasias, which have etiologies with and without HPVs such as anal and oral cancer (Hernandez et al. 2012; Jitesh et al. 2013). Although the cellular methylome of the same group of tumors may differ in the presence and the absence of HPVs (Sartor et al. 2011), there is no a priori need to assume that methylation may be affected by the functions of HPV gene products. Nevertheless, this may yet be the case, as the HPV-16 E7 oncoprotein was reported to associate in vitro and vivo with the DNA methyltransferase DNMT1 and to stimulate its activity (Burgers et al. 2007). This observation opens up the possibility that this epigenetic effect directly influences cellular proliferation pathways. Subsequent studies proposed as a consequence of this mechanism suppression of E-cadherin expression and reduced adhesion between squamous epithelial cells (Laurson et al. 2010; D'Costa et al. 2012) and extended the effect to interactions of both E6 and E7 protein with components of the histone modification machinery (Bodily et al. 2011; Hsu et al. 2012).

1.6 Differential Methylation of HPV Genomes in Malignant Lesions

It is known since the early days of HPV research in the 1980s that HPV genomes in cancer frequently exist in a form recombined with cellular DNA (Schwarz et al. 1985). It is now generally accepted that the transition from high-grade precursors (CIN III) to invasive carcinomas is accompanied by and possibly caused by this recombination (Mine et al. 2013), although it is still disputed whether all or only a subset of cancerous lesions contain HPV genomes in chromosomally recombined form (Kalantari et al. 2001; Peitsaro et al. 2002; Arias-Pulido et al. 2006;

Bhattacharjee and Sengupta 2006; Brandsma et al. 2009; Snellenberg et al. 2012; Chaiwongkot et al. 2013; Mirabello et al. 2013; Bryant et al. 2014). Recombination can result in interruption of the early polycistronic E6-E7-E1-E2 transcription unit. Failure to express E2 stimulates oncoprotein expression due to a lack of negative feedback repression of E2 on the E6 promoter. Beyond this, mechanisms for eliminating remaining episomal HPV genomes have recently been proposed as essential for cervical carcinogenesis (Mine et al. 2013).

In malignant and high-grade premalignant lesions, likely due to recombination with the cellular chromosomes, HPV genomes clearly undergo substantial methylation beyond the levels observed for episomal genomes (exceeding for some CpG residues 50%) as confirmed for HPV16 (Kalantari et al. 2004, 2014; Bhattacharjee and Sengupta 2006; Brandsma et al. 2009; Sun et al. 2011; Vinokurova and Knebel Doeberitz 2011; Xi et al. 2011; Clarke et al. 2012; Patel et al. 2012; Mirabello et al. 2013; Park et al. 2011; Verhoef et al. 2014; Frimer et al. 2015), HPV18 (Badal et al. 2004; Turan et al. 2006; Wentzensen et al. 2012; Kalantari et al. 2014; Vasiljevic et al. 2014), HPV31 (Wentzensen et al. 2012; Kalantari et al. 2014; Vasiljevic et al. 2014), HPV33 (Vasiljevic et al. 2014), HPV45 (Wentzensen et al. 2012; Kalantari et al. 2014), HPV52, and HPV58 (Murakami et al. 2013). For HPV16, this was reported not only for cervical but also vulval (Bryant et al. 2014), penile (Kalantari et al. 2008b), oral (Balderas-Loaeza et al. 2007), and anal cancer (Wiley et al. 2005; Hernandez et al. 2012). Methylation is relatively low in the LCR (which, together with the use of methylation sensitive restriction enzymes as opposed to bisulfite sequencing, led to an original misinterpretation of this mechanism, Badal et al. 2003), and is highest at certain CpGs in the late genes L2 and L1 (Brandsma et al. 2014; Mirabello et al. 2015).

Findings of increased methylation of HPV genomes correlating with the increasing severity of the lesion (from CIN I through CIN III to invasive cancer) were surprising and against intuition, as DNA methylation is normally seen as a transcription repression mechanism. The resolution of this contradiction came from two sources. Van Tine et al. (2004) reported in situ studies that cervical tumors typically contain numerous (i.e., up to a few hundred) HPV genome copies. All of these viral genomes are transcriptionally inactive, except one, which is the only source of E6 and E7 oncogene transcripts. In other words, some selective methylation mechanism targets these recombinant HPV genomes. Should all of them become methylated, HPV transcription would end, and such a clone would never grow into a detectable tumor. Only cells with one or few transcriptionally active HPV genomes grow into a detectable lesion.

This mechanism was further confirmed with the study of two cervical cancer cell lines, SiHa and CaSki. SiHa cells contain a single chromosomally recombined HPV16 genome, whose LCR is unmethylated and therefore transcriptionally active. CaSki cells contain about 500 HPV16 genomes, but generate a similar level of transcripts as SiHa cells. Not surprisingly, all HPV16 genomes in CaSki cells except one are methylated and transcriptionally inactive, oncogene transcripts being generated from the only unmethylated viral genome (Kalantari et al. 2004). However, it is not a necessary condition that most HPV genomes become

methylated. The well-known cell line HeLa had been derived from a cervical adenocarcinoma and was shown to contain about 50 chromosomally recombined copies of HPV18 DNA. The analysis of its HPV18 genomes showed that the LCR and the E6 gene are generally not methylated and remain transcriptionally active (Johannsen and Lambert 2013), while parts of the genome that are upstream of the LCR, such as the L1 genes are heavily methylated (Turan et al. 2007).

It is unknown why chromosomally recombined HPV genomes become preferentially methylated. HPV DNA may be targeted by a methylation mechanism affecting all foreign DNA in mammalian cells (Dörfler et al. 2001). More recently a view emerged that the methylated state of DNA may be quite in general the default state of the hosts chromosomal DNA to lock genes in an off position (Edwards et al. 2010; Schuebeler 2015).

1.7 HPV Epigenomes and Cellular Epigenomic Properties of HPV Infected Cells as Cancer Biomarkers

Cancer of the cervix affects about 500,000 women every year, and about half of these die of this disease. It is the most prevalent cancer in women in many developing nations, but its incidence has been reduced in developed nations, to a large part through early diagnosis of precancerous lesions and surgical intervention. From the 1950s to the 1990s, diagnosis was mostly based on the Papanicolaou test (Pap test), which can be complemented with colposcopic observation of lesions. The Pap test is a staining test of a cervical smear obtained during a gynecological examination, which was developed without knowledge of the viral etiology of cervical cancer. The Pap test is a tremendous public health success, but it is less than satisfactory as it has a high rate of false negative diagnoses, as it misses many lesions. Since HPV infections are the sole underlying cause of precancerous cervical neoplasia, HPV DNA detection has become a valuable tool to amend or replace the Pap test. However, many women are carriers of HPV infections, which never progress toward malignancies. At this time, the best practice is to administer both a Pap test and an HPV DNA test on a patient, as well as interpreting the outcome in the context of the age and the previous diagnostic history of the patient (Saslow et al. 2012).

From these considerations, it is obvious that the triage of women with a positive Pap test or positive for HPV infection would benefit from the development of tests based on novel biomarkers. Detection of DNA methylation has the potential to be such a biomarker, whose detection can be technically standardized and made capable for high-throughput processing. This chapter has discussed that HPV DNA is either unmethylated or lowly methylated in asymptomatic infections and precancerous CIN I lesion while heavily methylated in cancer, an increase that begins in high-grade precursor lesions (CIN III). Methylation is particularly high at certain CpG dinucleotides in the late genes L1 and L2, identifying the best targets

for HPV methylation analysis (Brandsma et al. 2009). A highly sensitive detection of these methylation changes may help to separate patients with malignantly progressing cervical lesions from those not undergoing such changes, as evaluated recently (Brandsma et al. 2014). In order to eliminate the time consuming DNA sequencing, HPV18 DNA methylation could be efficiently detected by PCR with methylation specific primers or with real-time PCR (Turan et al. 2007). As an alternative improvement toward clinical application, it has been shown that next-generation sequencing allows the establishment of the whole HPV16 methylome and eliminates laborious purification of PCR amplicons. Alternatively, pyrosequencing can target segments of HPV genomes of specific relevance for diagnosis. The same publication confirmed that high-grade precursors had a higher methylation than low-grade precursors, the decisive criterion for the usefulness to detect lesions likely to progress toward cancer (Mirabello et al. 2015). Beyond the analysis of the HPV genome, specific cellular genes such as DAPK and RARB are frequently methylated in cervical cancer (Wentzensen et al. 2009), and it may strengthen epigenomic testing to combine the measurement of HPV DNA methylation with that of the methylation status of such cellular genes (Sartor et al. 2011; Johannsen and Lambert 2013; Kalantari et al. 2014; Louvanto et al. 2015; Siegel et al. 2015). At this point, the utility of HPV methylation deserves to be further studied as a strategy to identify women at high risk for cervix cancer.

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Chapter 2
Epigenetic Regulation of Gammaherpesviruses: A Focus on Kaposi's Sarcoma-Associated Herpesvirus (KSHV/HHV-8)

Rosha Poudyal, Rolf Renne, and Michael P. Kladde

Abstract Gammaherpesviruses are ubiquitous in nature and infect a broad range of animal species. They have a biphasic life cycle that alternates between latent and lytic phases and are able to maintain a persistent infection for long periods. Both Epstein–Barr virus (EBV) and Kaposi's Sarcoma-associated Herpesvirus (KSHV) are oncogenic viruses that are known to cause several lymphoproliferative diseases in humans. EBV and KSHV persist as viral episomes that orchestrate very tightly controlled programs of gene expression, whereby a distinct subset of viral genes is expressed during the latent phase. Various stimuli can induce lytic reactivation of both viruses, which results in expression of lytic genes and is accompanied by changes in histone modification and DNA methylation. CTCF and cohesin binding provide segregation of chromatin loops as well as cross talk between different regions of the genome. Furthermore, noncoding RNAs (ncRNAs) provide an additional layer of epigenetic regulation for gammaherpesviruses. MAPit, a single-molecule footprinting assay, has revealed the occurrence of several subtypes of chromatin architecture at various KSHV promoters, suggesting the presence of heterogeneity within the population of KSHV viral episomes. In this chapter, we discuss the epigenetic regulation of gammaherpesviruses during latency and lytic reactivation, with a primary focus on KSHV.

Keywords Gammaherpesviruses • KSHV • EBV • Epigenetics • DNA methylation • Histone modifications • Epigenetic heterogeneity • CTCF • Cohesin • Non-coding RNA • microRNA • lncRNA • PAN RNA

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2.1 Introduction

Herpesviruses are ubiquitous, linear, double-stranded DNA viruses that infect a wide range of host animal species. More than 100 herpesviruses have been identified to date, which are classified into α, β, or γ subfamilies based on their genome organization and unique biological properties such as tissue tropism (Roizman 1982). Herpesviruses have a genome size ranging from 120 to 230 kilobases (kb), encoding 70–200 genes. Their genome is encapsulated in an icosahedral nucleocapsid that is approximately 100 nm in diameter (Liu and Zhou 2007). Herpesviruses establish a persistent infection and alternate between latent and lytic phases, exhibiting a gene expression pattern specific to each state. The γ-subfamily of herpesviruses is known to be oncogenic and is causally associated with several cancers, primarily in immunocompromised individuals. The γ-subfamily of herpesviruses is lymphotropic, but some have the capability to replicate in epithelial as well as endothelial cells. The two species of γ herpesviruses that infect humans are Epstein–Barr virus [EBV, also called Human Herpesvirus type 4 (HHV-4)] and Kaposi's Sarcoma-associated Herpesvirus (KSHV, also called HHV-8). EBV, discovered in 1964, infects approximately 90% of the adult world population and is largely associated with Hodgkin's lymphoma, Burkitt's lymphoma, and nasopharyngeal carcinoma (Epstein et al. 1964; Wei and Sham 2005; Maeda et al. 2009). KSHV, which was identified as the etiological agent of Kaposi's sarcoma (KS) in 1994, shows a diverse range of seroprevalence that varies among geographic regions (Table 2.1) (Cohen 2000; Chang et al. 1994; Chatlynne and Ablashi 1999; Wawer et al. 2001; Mohanna et al. 2005). KSHV has also been shown to cause two other neoplasms: Primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) (Cesarman et al. 1995; Soulier et al. 1995).

Table 2.1 Worldwide seroprevalence of human γ-herpesviruses and their associated disorders

γ-herpesvirus	Worldwide seroprevalence	Associated disease
EBV (HHV-4)	>90% in adult population	Burkitt's lymphoma Central nervous system lymphomas Gastric carcinoma Hodgkin's lymphoma Nasopharyngeal carcinoma Non-Hodgkin's lymphoma Post-transplant lymphoproliferative disorders T-cell lymphoma
KSHV (HHV-8)	40% seroprevalence in sub-Saharan Africa 10% in Mediterranean countries 2–4% in northern Europe, Southeast Asia, and the Caribbean 5–20% in the United States	Classical, endemic, and AIDS-related Kaposi's Sarcoma Multicentric Castleman's disease Primary Effusion Lymphoma

2.1.1 Genome Organization and Circularization

The genomes of EBV (~172 kb) and KSHV (~140 kb) within their viral icosahedral capsids are linear, double-stranded DNA molecules flanked by terminal repeats (TRs) that are used for circularization of the virus. EBV, a *Lymphocryptovirus*, has a variable repeated 500 bp sequence TR and also contains multiple internal repeats interspersed within the unique regions (Young et al. 2007). KSHV, a *Rhadinovirus*, has a long unique region that is flanked by a variable number of 801 bp long TRs (Renne et al. 1996a). The TRs harbor sequences that serve as an origin of replication (Renne et al. 1996a; Zimmermann and Hammerschmidt 1995). In the capsid, both EBV and KSHV have no detectable levels of DNA methylation and core histones (Johannsen et al. 2004; Birdwell et al. 2014; Bechtel et al. 2005).

During primary infections, EBV viral particles bind to host cell surface receptor CD21, whereas KSHV binds to integrin and Ephrin A2, and are then internalized by endocytosis. The viral capsids, which house the viral genomes, are trafficked through the cytoplasm to the perinuclear region, where the viral genomes are ejected into the nucleus through nuclear pores (D'Addario et al. 2001; Akula et al. 2002; Chakraborty et al. 2012; Hahn et al. 2012). This mode of genome delivery prevents viral DNA from being degraded and exposed to DNA-dependent activators in the cytoplasm (Chandran 2010). Upon entering the host nucleus, the viral DNA circularizes by recombination within the TRs (Fig. 2.1). Genome circularization is an essential step for efficient viral infection as linear genomes are subject to exonucleolytic attack and can activate the host DNA damage response pathway (Weitzman et al. 2010; Deng et al. 2012). After circularization, the viral DNA of both EBV and KSHV become "chromatinized," acquiring histones and subsequently persisting in the nucleus of the host as multicopy, closed-circular, extrachromosomal episomes (Fig. 2.1) [reviewed in Knipe et al. (2013)]. These nuclear episomes have similar attributes to host cellular chromatin and are packaged into nucleosomes with a characteristic repeat length [reviewed in Knipe et al. (2013)].

The mechanism by which the viral DNA duplex establishes a successful nonreproductive, latent episomal state and is reactivated to a productive lytic phase is poorly understood. The genomes of both EBV and KSHV code for several proteins involved in immune evasion and cell cycle regulation, some of viral origin as well as copies of pirated cellular homologs (Ressing et al. 2015; Lee et al. 2012). In addition, both viruses encode microRNAs (miRNAs) and long noncoding RNAs (lncRNAs). The EBV genome contains about 80 protein coding genes, with two microRNA clusters within the *BART* and *BHRF1* genes [reviewed in Skalsky and Cullen (2015)]. KSHV has the coding potential for nearly 86 genes, 18 mature microRNAs from the KSHV latency-associated region (*KLAR*), and several noncoding RNAs [reviewed in Zhu et al. (2014)].

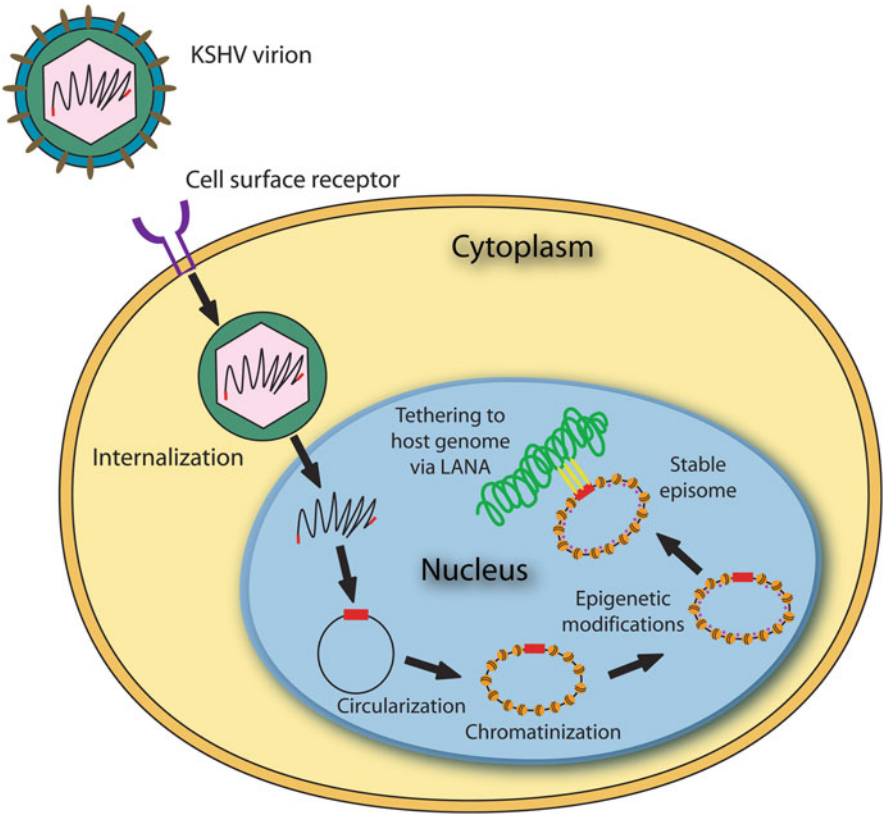

Fig. 2.1 Establishing a latent KSHV infection. During de novo infection, KSHV virions bind to the cell surface receptors, Integrin and Ephrin A2, and enter the cytoplasm by endocytosis. The viral capsid containing the linear, dsDNA viral genome docks with the nuclear pore and ejects the viral genome into the nucleus. Once inside the nucleus, the virus circularizes at its terminal repeats (TRs; *red rectangle*). During replication, the KSHV genome is assembled into nucleosomes, and cellular chromatin-modifying machinery is recruited to posttranslationally mark core histones and establish episomes that stably replicate as extrachromosomal minichromosomes. Methylation of DNA at inactive promoters and other regions is apparently established over the course of many months post infection. KSHV LANA protein (*yellow ellipses*) binds to the TRs and tethers the viral genome to host chromosomes. Viral episomes replicate synchronously with the host genome during S phase and are segregated to daughter cells during cell division, thereby enabling a persistent infection

2.1.2 Gene Expression During Latency

Gammaherpesviruses exhibit a biphasic life cycle with a persistent, but reversible, latent phase and a transient lytic reactivation phase. Both phases are characterized by distinct, tightly regulated gene expression profiles that are governed through the concerted action of histone modifications, DNA methylation, and noncoding RNAs (Tsurumi et al. 2005; Dourmishev et al. 2003). EBV displays more than one type of

latency program, namely, Latency 0, Latency I, Latency II, and Latency III. Each of the latency programs is associated with the expression of a limited and distinct set of viral proteins and can vary in different cell types (Amon and Farrell 2005). Latency 0, observed in noncycling and resting B cells, is the most tightly regulated transcription program, where no viral genes are transcribed (Babcock et al. 2000). On the other hand, Latency III, observed in highly proliferating B cells, is the most transcriptionally permissive program, expressing all the gene products associated with latency, such as EBV nuclear antigen 1 (*EBNA1*), *EBNA2*, as well as latent membrane protein 1 (*LMP1*), *LMP2A*, and *LMP2B* [reviewed in Young and Rickinson (2004)].

KSHV may display less variation in latency types than EBV. Upon de novo infection, genes responsible for viral latency are expressed from the *KLAR* as a multicistronic transcript that encodes latency-associated nuclear antigen (LANA), virus-encoded Cyclin D homolog (vCyclin), viral Fas-associated death domain-like interleukin-1 beta-converting enzyme (FLICE)-inhibitory protein (vFLIP), and the Kaposin (K12) family of proteins (Zhong et al. 1996; Dittmer et al. 1998). No infectious viral particles are produced during latency. LANA, a functional ortholog of EBNA1 with respect to latent DNA replication and episome tethering, binds preferentially to the TRs of the viral genome and tethers it to the host chromosome. This ensures the faithful segregation of the viral genome to host daughter cells during cell division (Ballestas et al. 1999; Cotter and Robertson 1999). In addition, LANA is a multifunctional protein that is known to either activate or repress transcription of various cellular and viral genes (Renne et al. 2001; Garber et al. 2001; Fujimuro et al. 2003). The other *KLAR* gene products play key roles in host cell proliferation and survival (Chang et al. 1996; Thome et al. 1997; Ye et al. 2008). The chromatinization that leads to a condensed form of the episome provides protection to the viral genome from degradation while enabling tight regulation of gene expression [reviewed in Lieberman (2013)].

2.1.3 Gene Expression During Lytic Phase

For the completion of a full life cycle and maintenance of a persistent infection, gammaherpesviruses are required to undergo reactivation from latency, whereby new infectious viral particles are produced during lytic replication. Although the stimuli that promote the switch from latency to the lytic phase are not completely understood, various cellular phenomena, such as immune suppression, oxidative stress, and hypoxia, are known to trigger lytic reactivation [reviewed in Ye et al. (2011)]. In vitro, cells that are latently infected with EBV, KSHV, or both can be reactivated when treated with drugs that alter epigenetic modifications, e.g., sodium butyrate, a histone deacetylase inhibitor (HDACi), or 5-aza-2′-deoxycytidine, a DNA demethylating agent (Knipe et al. 2013; Shin et al. 2014; Shamay et al. 2006). During reactivation, the viral DNA is replicated by a viral polymerase along with a timely regulated cascade of gene expression, leading to the assembly and egress of

mature infectious virions. Lytic viral genes are expressed in temporal fashion, activating three classes of lytic genes: immediate-early (IE), early (E), and late (L) genes (Renne et al. 1996b; Sun et al. 1999; Jenner et al. 2001).

The key viral IE proteins that are required for the transcriptional activation of other lytic genes are Zta in EBV and Replication and Transcription Activator (RTA/ORF50) in KSHV (Gl et al. 2007). Although a number of IE-lytic genes, such as *RTA*, *ORF45*, *K8.2*, *K4.2*, etc., are expressed upon reactivation in KSHV, it has been established that *RTA* functions as the master switch between latent and lytic gene expression (Sun et al. 1998). RTA is the only lytic viral protein that is both necessary and sufficient for the activation of several lytic promoters and replication of the viral genome (Wang et al. 2003; Guito and Lukac 2012). RTA is also known to auto-activate its own promoter through an RTA-responsive element and establish a positive feedback loop in the viral lytic gene expression (Deng et al. 2000). The expression of E genes is activated by IE gene products, which mostly include proteins that have enzymatic functions that are required for DNA replication (e.g., DNA polymerase I processivity factor ORF59) and for modulation of the immune system (MIR1/2) (Coscoy and Ganem 2001; Ishido et al. 2002; Majerciak et al. 2006). The L genes are expressed following the expression of E-lytic genes and are transcribed after lytic DNA replication (Honess and Roizman 1974). The L-lytic gene products consist of several viral structural proteins, including major capsid protein (MCP) encoded by ORF25, several membrane glycoproteins (K8.1), and a viral capsid antigen that facilitates the assembly and maturation of virions (Schulz and Yuan 2007). Viral tegument proteins that assist the virus during virion assembly, viral entry, and host immune evasion are also a part of the L-lytic gene expression period [reviewed in Sathish et al. (2012)].

2.2 Histone Modifications

Genomic DNA in the nucleus of all eukaryotic cells is associated with core histone proteins and nonhistone regulatory proteins to form chromatin. The fundamental repeating unit of chromatin, the nucleosome, comprises a histone octamer of two copies of each core histone protein (H2A, H2B, H3, and H4) wrapped by a left-handed superhelix of 147 bp of DNA, plus 20–80 bp of linker DNA (Li and Reinberg 2011; Kornberg and Lorch 1999; Luger 2003). Nucleosomes are organized in arrays and display higher levels of folding/condensation and chromatin organization.

The N-terminal tails of a histone octamer, which protrude from the globular domain of the nucleosome, can undergo at least eight distinct types of posttranslational covalent modification (Kouzarides 2007). An increasing number of amino acid residues (arginine, lysine, serine, and threonine) in the histone N-terminal tails are subject to posttranslational modifications, including acetylation, methylation, phosphorylation, ubiquitination, and ADP-ribosylation. Furthermore, the local concentration of differentially modified nucleosomes allows for the regulation of