Chapter 8
Epigenetic Changes in Virus-Associated Neoplasms

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Abbreviations

5-caC	5-Carboxylcytosine
5-hmC	5-Hydroxymethylcytosine
5-mC	5-Methylcytosine
AIDS-BL	AIDS-related-BL
APC	Adenomatous polyposis coli
BART	BamHI A rightward transcripts
BCBL	Body cavity-based lymphoma
BL	Burkitt's lymphoma
CBF1	C promoter-binding factor 1
CGI	CpG island
cHL	Classical Hodgkin's lymphoma
CIMP	CpG island methylator phenotype
CIN	Cervical intraepithelial neoplasm
CIN	Chromosomal instability
Cp	C promoter
CpG	Cytosine-phosphate-guanine dinucleotide
CRBP	Cellular retinol-binding protein
CTCF	CCCTC-binding factor
DLBCL	Diffuse large B-cell lymphoma
DNMT	DNA methyltransferase
DS	Dyad symmetry

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J. Minarovits and H.H. Niller (eds.), *Patho-Epigenetics of Disease*,
DOI 10.1007/978-1-4614-3345-3_8,

EBER	Epstein–Barr encoded small RNA
eBL	Endemically occurring BL
EBNA	Epstein–Barr nuclear antigen
EBNA-LP	EBNA-leader protein
EBV	Epstein–Barr virus
EZH2	Enhancer of zeste homologue 2
FR	Family of repeats
GC	Gastric carcinoma
GC	Germinal center
GSTP1	Glutathione S-transferase P1
H3K27me3	Histone 3 trimethylated on lysine 27
H3K4me2	Histone 3 dimethylated on lysine 4
H3K4me3	Histone 3 trimethylated on lysine 4
H3K9me3	Histone 3 trimethylated on lysine 9
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDAC	Histone deacetylase
HHV-8	Human herpesvirus-8
HL	Hodgkin's lymphoma
HP1	Heterochromatin-associated protein 1
HPV	Human papillomavirus
HRS	Hodgkin and Reed–Sternberg cells
HTLV	Human T-lymphotropic virus
IM	Infectious mononucleosis
KDM	Lysine demethylase
KSHV	Kaposi's sarcoma herpesvirus
LANA	Latency-associated nuclear antigen
LCL	Lymphoblastoid cell line
LCR	Locus control region *or* long control region
LINE-1	Long interspersed element-1
LMP	Latent membrane protein
LMP2Ap	LMP2A promoter
lncRNA	Long noncoding RNA
MCD	Multicentric Castleman's disease
MCPyV	Merkel cell polyomavirus
MeCP2	Methylcytosine-binding protein 2
MGMT	O6-methylguanine DNA methyltransferase
miRNA	MicroRNA
NHL	Non-Hodgkin lymphoma
NPC	Nasopharyngeal carcinoma
PAN	Polyadenylated nuclear RNA
PcG	Polycomb group
PEL	Primary effusion lymphoma

PIN	Prostatic intraepithelial neoplasias
pRB	Retinoblastoma protein
PRC	Polycomb repressive complex
PTEN	Phosphatase and tensin homologue
PTLD	Posttransplant lymphoproliferative disorder
Qp	Q promoter
RARβ2	Retinoic acid receptor β2
RARRES	Retinoic acid receptor responder
RASSF1A	RAS association domain family 1 isoform A
sBL	Sporadic BL
SFRP1	Secreted frizzled-related protein 1
siRNA	Short interfering RNA
snoRNA	Small nucleolar RNA
SOCS	Suppressor of cytokine signaling
TAg	T antigen
TR	Terminal repeat
TrxG	Trithorax group
Wp	W promoter

8.1 Introduction

Viruses are associated with a significant fraction of neoplasms in mammals. Similarly to the malignant tumors elicited by other agents and the so-called spontaneous neoplasms of unknown etiology, the carcinomas, leukemias, lymphomas, and sarcomas induced by oncoviruses also frequently accumulate both genetic aberrations and epigenetic changes. It is well documented that infection by oncoviruses may introduce new information into the host cell DNA and change the structure and expression pattern of the cellular genome. DNA virus genomes or DNA copies of retrovirus (RNA tumor virus) genomes may either act as insertional mutagens that inactivate cellular genes, or may affect the regulation and expression of key cellular genes by the mechanism of promoter insertion or enhancer insertion. Furthermore, certain oncoproteins encoded by tumor viruses may directly elicit mitotic disturbances resulting in the generation of aneuploid cells or upregulate cellular enzymes with mutagenic activities.

Notwithstanding these remarkable observations, most researchers agreed that the main effect of the pleiotropic viral oncoproteins was either a direct, constitutive stimulation of cell proliferation or a continuous maintenance of cell growth in a less direct manner, via interfering with a series of tumor suppressor proteins involved in cell cycle regulation, apoptosis, and maintenance of genomic integrity. More subtle events attesting that epigenetic alterations, affecting both viral and cellular DNA sequences, also occur in transformed cells—see the review on oncogenic human adenoviruses by Walter Doerfler, Chap. 1—were not connected directly to the phenomenon of "malignant transformation" in the minds of most investigators.

Most recently, however, the focus of oncovirus research shifted, and a new concept, the epigenetic reprogramming of host cells by oncoproteins, gained momentum. Other tumor-associated pathogens, most notably *Helicobacter pylori*, a bacterium causing the majority of gastric cancer cases (see Chap. 14), and certain macroparasites, such as *Schistosoma haematobium* associated with bladder cancer (Gutierrez et al. 2004) and *Opisthorchis viverrini* (liver fluke) associated with cholangiocarcinoma (Chinnasri et al. 2009; Sriraksa et al. 2011), were also linked to the alterations of the host cell epigenome.

A recent comprehensive analysis of genome-wide DNA methylation patterns in a series of human neoplasms including colon carcinoma, lung carcinoma, breast carcinoma, thyroid carcinoma, and Wilms' tumor identified cancer-specific differentially DNA-methylated regions that lost the epigenetic stability characteristic to the corresponding normal tissues (Hansen et al. 2011). The loss of DNA methylation stability was associated with an increased gene expression variation. The major difference between normal and neoplastic tissues was the appearance of large, contiguous hypomethylated blocks in the analyzed carcinomas, although a small fraction of hypermethylated blocks were also detected. Hansen et al. suggested that the loss of epigenetic stability, i.e., increased CpG methylation variability and gene expression variability, might have a potential selective value in a changing environment. As formulated by Issa, an increased epigenetic plasticity may provide "a mechanism of Darwinian evolution at the cellular level that may underlie age-related diseases such as cancer" (Issa 2011).

The neoplasms studied by Hansen et al. are usually not regarded as virus-associated. Thus, the existence of epigenetically hypervariable regions remains to be demonstrated for virus-associated tumors. It is important to note, however, that regularly occurring stable epigenetic changes were also described both in virus-associated and non-virus-associated neoplasms. These recurrent, stable epigenetic alterations that are maintained during successive stages of tumor progression frequently affect the expression of a set of tumor suppressor genes or tumor-associated genes independently of the histological type of the neoplasm. Other stable epigenetic changes or their combinations appear to be specific for certain tumor types, or mark distinct stages of neoplastic development, and oncovirus-specific epigenetic signatures were also identified (reviewed by Niller et al. 2009). In this chapter, we focus almost exclusively on viruses associated with human neoplasms. Human viruses causing malignant tumors in experimental animals but not associated regularly with human neoplasms are either discussed in Chap. 1 (human adenoviruses) or described only briefly (human BK polyomavirus, this chapter, Sect. 8).

8.2 Epigenetic Alterations in Epstein–Barr Virus-Associated Neoplasms

Epstein–Barr virus (EBV), the first human "tumor virus" to be discovered, was observed initially by electron microscopy (Epstein et al. 1964) in cultivated cells derived from Burkitt's lymphomas (BLs, Burkitt 1958, 1962). For this historical

reason, and because EBV is one of the most comprehensively studied viruses with regard to the epigenetic regulation of the viral oncogenes and the pathoepigenetic consequences of viral infection, we start this chapter on oncovirus-associated dysregulation of the host cell epigenome with a brief description of the natural history and latency types of EBV (for more detailed reviews, see Niller et al. 2007, 2008).

8.2.1 *Epstein–Barr Virus: Basic Facts, Natural History, and Latency Types*

EBV is one of the eight known human pathogenic herpesviruses. The double-stranded linear DNA genome packaged into the virions of the prototype EBV strain B95-8 has a length of 172 kb pairs. Based on biological properties and sequence comparisons, EBV was classified as a member of the genus *Lymphocryptovirus* within the subfamily *Gammaherpesviridae* of the family *Herpesviridae*. The name *Lymphocryptovirus* refers to a fascinating property of EBV and other members of the genus: these viruses "hide" in lymphoid organs. This means that in addition to productive (lytic) replication that occurs in the epithelial cells of the oropharynx, EBV genomes can also persist for an extended period without the production of virions in latently infected host cells, notably within resting memory B lymphocytes. As we discuss it below, the expression of the EBV genome is highly restricted during latent infection in peripheral B cells, and in the virtual absence of viral protein expression (latency type 0, see Table 8.1), such EBV-infected B cells remain practically "invisible" for the immune system.

About 90% of the world's population is infected by EBV. The virus is intermittently shed into the saliva of persistently infected individuals, and the saliva is the main route of transmission to uninfected individuals. Although primary EBV infection is usually inapparent in early childhood, in adolescents or adults it may cause infectious mononucleosis (IM; also called glandular fever or kissing disease). Lymphadenopathy, an essential feature of IM, is a consequence of the proliferation of EBV-infected, activated B cells. In contrast to resting B cells, the EBV genome is not completely silenced in B lymphoblasts of IM patients, and the expressed viral oncoproteins (nuclear antigens, called EBNAs, and latent membrane proteins, LMPs; latency type III, see Table 8.1) stimulate continuous cell proliferation. Because most of the EBNAs as well as the LMPs are highly immunogenic, i.e., "visible" for the cells of the adaptive immune system, IM is usually curbed by a vigorous cellular immune response directed against latent EBV proteins. Latent, transcriptionally silent EBV genomes persist, however, lifelong in memory B cells.

EBV is associated with a series of malignant tumors including lymphomas (Burkitt's lymphoma, Hodgkin's lymphoma, T-/NK-cell lymphoma, posttransplant lymphoproliferative disease (PTLD), AIDS-associated lymphoma, X-linked lymphoproliferative syndrome), carcinomas (nasopharyngeal carcinoma (NPC), gastric carcinoma, carcinomas of the major salivary glands, thymic carcinoma, mammary carcinoma), and a sarcoma (leiomyosarcoma) (see Table 8.2). Strikingly, the incidence of Hodgkin's

Table 8.1 Latency types of Epstein–Barr virus

Latency type	Representative cell type	Active promoter	Expressed product
0	Resting B cell	Qp (?)	EBNA1 (variable?)
		LMP2Ap (?)	LMP2A (?)
I	Burkitt's lymphoma	Qp	EBNA1
		EBER1p	EBER1
		EBER2p	EBER2
		BARTp	BART, microRNAs processed from BART
II	Nasopharyngeal carcinoma	Qp	EBNA1
		EBER1p	EBER1
		EBER2p	EBER2
		BARTp	BART, microRNAs processed from BART
		LMP1p	LMP1 (variable)
		LMP2Ap	LMP2A
		LMP2Bp	LMP2B
GC	Gastric carcinoma	Qp	EBNA1
		EBER1p	EBER1
		EBER2p	EBER2
		BARTp	BART, microRNAs processed from BART
		LMP2Ap	LMP2A (variable)
		BARF1p	BARF1
III	Lymphoblastoid cell line	Cp	EBNA1-6, miR-BHRF1-3
		EBER1p	EBER1
		EBER2p	EBER2
		BARTp	BART, microRNAs processed from BART
		LMP1p	LMP1
		LMP2Ap	LMP2A
		LMP2Bp	LMP2B

Table 8.2 Epstein–Barr virus-associated neoplasms

Lymphomas
Burkitt's lymphoma
Hodgkin's disease
T-/NK-cell lymphoma
Posttransplant lymphoproliferative disease
AIDS-associated lymphoma
X-linked lymphoproliferative disease
Lymphomas in methotrexate-treated rheumatoid arthritis and polymyositis patients
Carcinomas
Nasopharyngeal carcinoma
Gastric carcinoma
Carcinomas of the major salivary glands
Thymic carcinoma
Mammary carcinoma
Sarcoma
Leiomyosarcoma

Table 8.3 Epigenetic regulation of latent Epstein–Barr virus promoters

		Epigenetic mark		
Promoter	Activity state	CpG methylation	Histone acetylation	H3K4me2
Qp	On	–	+	+
	Off	–	+	+
Cp	On	–	+	+
	Off	+	–	+/–
Wp	On	+		
	Off	–		
EBER1p	On	–		
	Off	?		
EBER2p	On	–		
	Off	?		
BARTp	On	(–)		
	Off	?		
LMP1p	On	–		
	Off	–		
LMP2Ap	On	–	+	+
	Off	+	–	+/–

lymphoma (HL) is increased after passing through symptomatic primary infection, i.e., IM (Niller et al. 2011), and the incidence of multiple sclerosis is increased after EBV infection by itself and even higher after IM, in addition (Niller et al. 2008).

The expression pattern of latent EBV genomes depends on the host cell phenotype. In vivo, BL cells express only a single EBV-encoded nuclear antigen, EBNA1, a transcription and replication factor (latency type I, see Table 8.1). In addition, two EBV-encoded small RNAs (EBER1 and 2) and a family of multiply spliced transcripts encoded by the *Bam*HI A fragment of the viral genome (BARTs, *Bam*HI A rightward transcripts) are also transcribed in BLs. BARTs potentially code for proteins, but they are also processed to viral microRNAs influencing the level of both viral and cellular mRNAs. In HL and NPC, latent membrane proteins (LMP1, LMP2A, LMP2B) can also be detected in addition to EBNA1, EBERs, BARTs, and viral microRNAs (latency type II, see Table 8.1). LMP1 is an oncoprotein contributing to apoptosis resistance of the infected cell, whereas LMP2A enhances metastasis formation. A unique latency type is characteristic for EBV-associated gastric carcinoma (GC) cells. Although the expression pattern is similar to that of latency type I cells, in addition to the typical latency type I EBV products, BARF1, originally characterized as a lytic cycle protein, and—variably—LMP2A are also expressed in GCs (Tables 8.1 and 8.3).

Whereas in all of the neoplasms (lymphomas and carcinomas) described above EBNA1 transcripts are initiated at Qp, a promoter located to the *Bam*HI Q fragment of the EBV genome, expanding B cells of PTLD and in vitro immortalized B lymphoblastoid cell lines (LCLs) use a B lymphoblast-specific promoter, Cp, to initiate transcripts coding for 6 nuclear antigens (EBNA1-6; Table 8.3). This expression pattern is similar to that of the proliferating B cells of IM patients (latency type III, Table 8.1). Similarly to the BARTs, viral microRNAs are also processed from the

EBNA transcripts initiated at Cp. Since EBNA2, the major transactivator protein of EBV, switches on the expression of the LMP1, LMP2A, and LMP2B genes, PTLDs and LCLs regularly express latent membrane proteins. In other LMP-expressing cell types, cellular transcription factors may substitute for EBNA2 and switch on the LMP promoters.

8.2.2 Epigenotypes of Latent Epstein–Barr Virus Genomes

The cell type-specific expression of latent EBV genes is achieved by epigenetic regulatory mechanisms controlling the activity of the alternative promoters Cp and Qp, and a third promoter for EBNA1-6 transcripts, Wp, that is less frequently used in tumor cells. The very same epigenetic regulatory mechanisms (DNA methylation, histone modifications, protein–DNA interactions) determine the activity of the promoters for LMP1, LMP2A, and LMP2B transcripts as well.

DNA methylation at position 5 of cytosine is involved in transcriptional silencing via the establishment of a "closed" chromatin structure suppressing transcription. DNA methylation patterns are *maintained* by DNA methyltransferase 1 (DNMT1) that restores the methylation pattern of the parental strands on the initially unmethylated daughter strands during DNA replication, whereas other DNMTs (DNMT3A, DNMT3B) can act on completely unmethylated DNA strands (de novo DNA methyltransferases, see Chap. 1). DNA methylation is reversible: one could distinguish active and passive mechanisms of DNA demethylation. The recently explored active pathway proceeds through conversion of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) and further to 5-carboxylcytosine (5-caC) by the Tet family of dioxygenases, followed either by decarboxylation (Ito et al. 2011) or excision by thymine-DNA glycosylase that triggers the base excision repair pathway replacing thereby 5-mC with C (He et al. 2011). The passive pathway involves the inhibition of DNMT1 activity during two successive cell cycles that may result in hemimethylated and finally completely unmethylated DNA stretches.

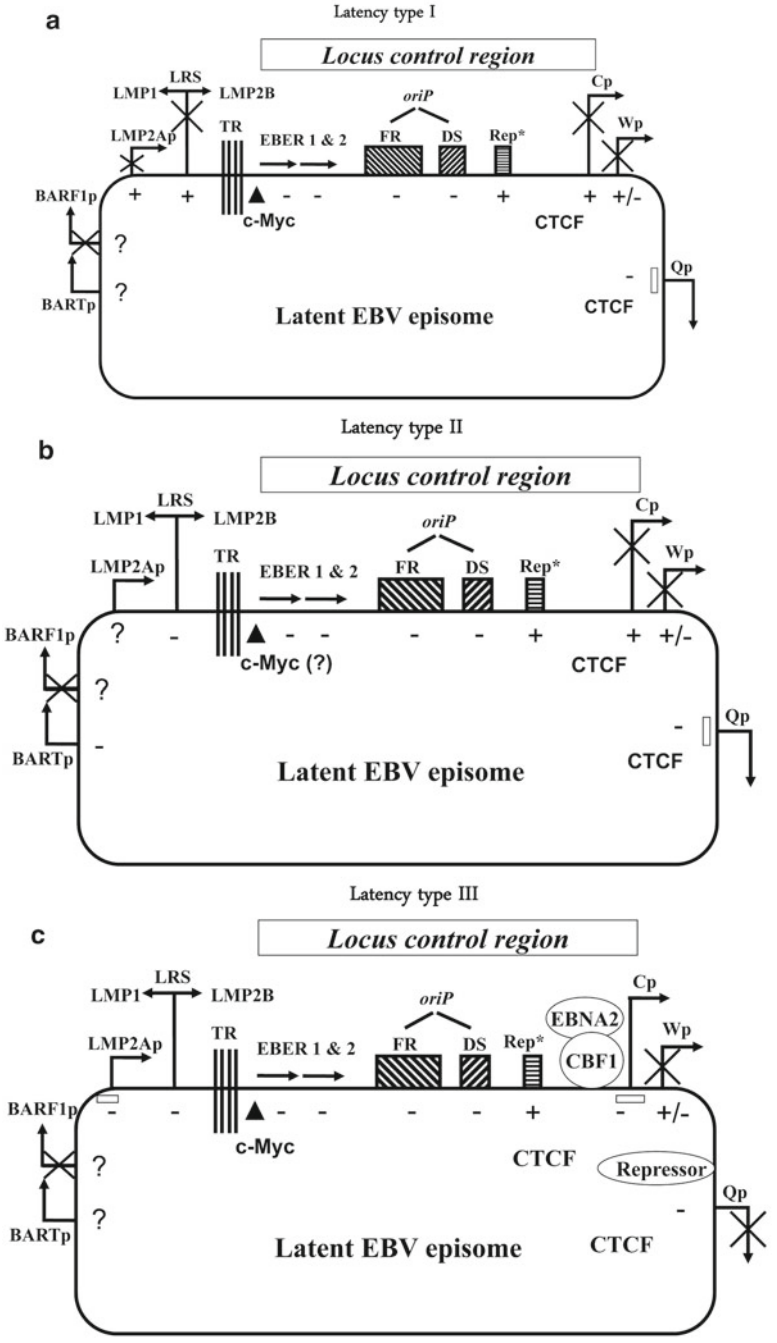
The methylcytosine-binding protein 2 (MeCP2) can attach to hypermethylated DNA sequences with high affinity and attract histone deacetylases that remove the acetyl moieties of histone tails, thereby eliciting chromatin compaction (Nan et al. 1998b). Thus, silent promoters are frequently associated with histones H3 and H4 that are devoid of acetylation. In contrast, active promoters are frequently unmethylated, and they are associated with an "open" chromatin configuration, due to the action of histone acetyltransferases that enrich the chromatin in acetylated histones H3 and H4, thereby creating so-called acetylation islands (Roh et al. 2005). DNA methylation regulates the activity of latent EBV promoters Cp, Wp, LMP1p, LMP2Ap, and BARTp (Table 8.3). Acetylation islands were identified at the active EBV latency promoters Cp, Qp, and LMP2Ap (Table 8.3).

DNA methylation patterns are transmitted from cell generation to cell generation (epigenetic memory). An alternative system of epigenetic memory, the Polycomb–Trithorax group (TrxG) of protein complexes, also ensures a heritable regulation of gene expression. Polycomb group (PcG) protein complexes can silence promoters.

The histone lysine methyltransferase enhancer of zeste homologue 2 (EZH2), a member of the Polycomb repressive complex 2 (PRC2), trimethylates lysine 27 of histone H3, thereby producing a repressive histone mark (H3K27me3), whereas PRC1 has a histone ubiquitinase activity (reviewed by Ringrose and Paro 2007; Blomen and Boonstra 2011). The repressive mark H3K27me3 left on the chromatin by PRC2 serves as a recruitment site for PRC1. In contrast to PcG protein complexes that silence promoters, histone lysine methyltransferase members of the TrxG protein complex create an activating histone modification (H3K4me3, histone H3 trimethylated on lysine 4). It is interesting to note that certain histone lysine demethylases, which are also components of the TrxG complex, actively remove the repressive H3K27me3 mark left by PRC2. The antagonism is mutual because other histone lysine demethylases, associated with PcG complexes, remove the activating H3K4me3 mark (reviewed by Blomen and Boonstra 2011). The role of PcG protein complexes in the regulation of latent EBV promoters remains to be elucidated. The H3K4me3 mark left by the TrxG complex was identified, however, at the active promoters Cp, Qp, and LMP2Ap (Table 8.2). In summary, the latent EBV genomes carried by host cells as nuclear matrix-attached circular episomes are "decorated" with cell type-specific epigenetic marks. On this basis, one can distinguish between unique viral epigenotypes (Minarovits 2006). The epigenetic marks associated with latent EBV episomes or viral epigenotypes ensure differential expression of the identical or nearly identical viral genomes in various host cells. The best characterized epigenotypes of latent EBV genomes, that correspond to some of the major latency types, are shown in Fig. 8.1.

Whereas Fig. 8.1 depicts a linear model of the major EBV latency types based on the composition (i.e., epigenetic marks) of the chromatin fiber, recent data suggest that the 3D structure of chromatin may also influence the activity of EBV latency promoters. Tempera et al. described that distinct, alternative chromatin loops of the EBV episome could be detected in a latency type I BL cell line and a latency type III LCL (Tempera et al. 2011; Fig. 8.2). According to the chromatin conformational model of EBV latency, in the examined latency type I BL cell line, the dyad symmetry (DS) element and the family of repeats (FR) element of *oriP*, the latent origin of EBV replication, associate with Qp in concert with a binding site of the insulator protein CTCF located between *oriP* and Cp. CTCF plays an integral role in this interaction which results in the formation of a chromatin loop between Qp and *oriP* and activates the initiation of EBNA1 transcripts at Qp (Fig. 8.2a). In contrast, in a latency type III LCL, the DS element of *oriP* interacts with the CTCF-binding site situated between *oriP* and Cp, i.e., upstream of the C promoter. This interaction generates an alternative chromatin loop, in this case between *oriP* and Cp, and facilitates active transcription of the polycistronic mRNA coding for 6 EBNA proteins, at Cp (Fig. 8.2b) (Tempera et al. 2011). When independently confirmed, these data may help to build up cell type-specific 3D models of latent EBV episomes. Further studies may also help to firmly establish the relationship between alternative chromatin loops and transcriptional activity of EBV latency promoters, too.

Although Tempera et al. could successfully deplete CTCF in EBV-bacmid carrying HEK-293 cells using a short interfering RNA (siRNA) targeting CTCF (siCTCF), and thereby abolish the chromatin loop between Qp and *oriP*, this intervention had


a
Latency type I
Locus control region
LRS
LMP1
LMP2B
LMP2Ap
TR
EBER 1 & 2
oriP
FR
DS
Rep*
Cp
Wp
BARF1p
c-Myc
CTCF
Qp
BARTp
Latent EBV episome
b
Latency type II
Locus control region
c-Myc (?)
c
Latency type III
Locus control region
EBNA2
CBF1
Repressor

only a moderate impact on the activity of Qp and Cp. Accordingly, neither EBNA1 nor EBNA2 mRNA levels were significantly affected by siCTCF and the disruption of *oriP*–Qp interaction in HEK-293 cells (Tempera et al. 2011). It is also worthy to consider that Cp-reporter gene constructs lacking *oriP* but containing a binding site for the nuclear protein CBF1 (C promoter-binding factor 1/RBP-Jκ) are highly active in latency type III cells (Minarovits et al. 1994). Such constructs are apparently unable to form an intraepisomal *oriP*–Cp loop, suggesting that the presence of a large chromatin loop upstream of Cp is dispensable for Cp activity. In addition, in cell lines carrying latent EBV genomes, CTCF binds to its recognition sequence located upstream of Cp independently of Cp activity (Salamon et al. 2009). In cells actively using Cp, CTCF binding to this site does not appear to block the putative long-range enhancer activity of *oriP* (Salamon et al. 2009). It does not function as a "barrier insulator," either, because it does not prevent spreading of CpG methylation through the CTCF-bound sequence in cell lines carrying silent Cp (Salamon et al. 2009). It was also observed that CTCF binds to both silent and active Q promoters (Salamon et al. 2009). Thus, one may argue that CTCF binding to the EBV episomes does not necessarily influence the activity of the alternative promoters Cp and Qp. Based on these considerations, Takacs et al. suggested that CTCF might play a structural role in the physiology of latent EBV genomes by contributing to

Fig. 8.1 Epigenotypes of latent Epstein–Barr virus genomes. (**a**) Latency type I epigenotype. The circular episomal genome is shown with the latent viral promoters (*arrows*) and their regulatory regions (not to scale). An LCR involved in attachment to nuclear matrix includes *oriP*, the latent origin of virus replication that also acts as a long-range enhancer. *TR* terminal repeats; *c-Myc* a nuclear protein-binding upstream of EBER1 (as indicated by a *triangle*); *EBERs 1 and 2* transcription units for nontranslated viral RNA molecules; *FR, DS, Rep** elements involved in latent EBV replication; *CTCF* insulator protein. *Symbols*: "+" indicates a high level of regional CpG methylation, "–" indicates unmethylated or hypomethylated CpG dinucleotides; "crossed-out arrow," silent promoter; "open box," hyperacetylated island, a chromatin region favoring transcription. Qp, EBER1p, EBER2p, and BARTp are active. (**b**) Latency type II epigenotype. The circular episomal genome is shown with the latent viral promoters (*arrows*) and their regulatory regions (not to scale). An LCR involved in attachment to nuclear matrix includes *oriP*, the latent origin of virus replication that also acts as a long-range enhancer. *TR* terminal repeats; *c-Myc* a nuclear protein-binding upstream of EBER1 (as indicated by a *triangle*); *EBERs 1* and *2* transcription units for nontranslated viral RNA molecules; *FR, DS, Rep** elements involved in latent EBV replication; *CTCF* insulator protein. *Symbols*: "+" indicates a high level of regional CpG methylation, "–" indicates unmethylated or hypomethylated CpG dinucleotides; "crossed-out arrow," silent promoter; "open box," hyperacetylated island, a chromatin region favoring transcription. Qp, EBER1p, EBER2p, and BARTp, LMP1p, LMP2Ap, and LMP2Bp are active. (**c**) Latency type III epigenotype. The circular episomal genome is shown with the latent viral promoters (arrows) and their regulatory regions (not to scale). An LCR involved in attachment to nuclear matrix includes *oriP*, the latent origin of virus replication that also acts as a long-range enhancer. *TR* terminal repeats; *c-Myc* a nuclear protein-binding upstream of EBER1 (as indicated by a *triangle*); *EBERs 1* and *2* transcription units for nontranslated viral RNA molecules; *FR, DS, Rep** elements involved in latent EBV replication; *CTCF* insulator protein. *Symbols*: "+" indicates a high level of regional CpG methylation, "–" indicates unmethylated or hypomethylated CpG dinucleotides; "crossed-out arrow," silent promoter; "open box," hyperacetylated island, a chromatin region favoring transcription. Cp, EBER1p, EBER2p, and BARTp, LMP1p, LMP2Ap, and LMP2Bp are active

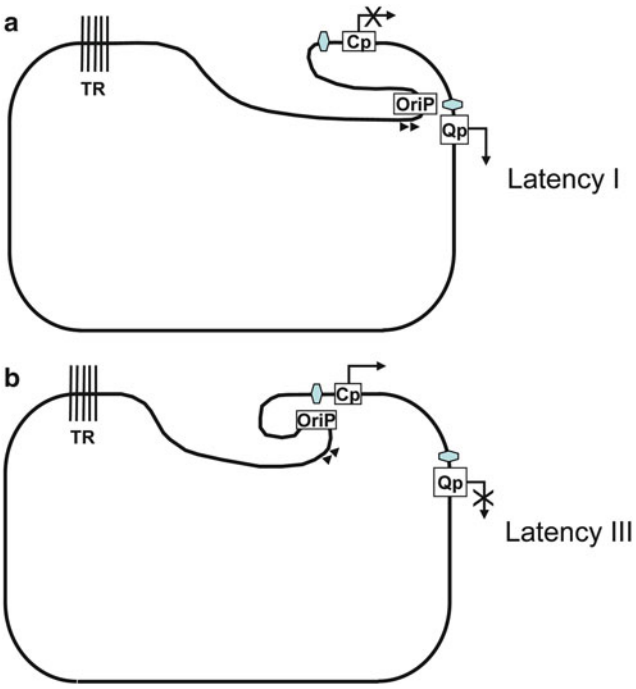


Fig. 8.2 A chromatin-looping model for the regulation of latent Epstein–Barr virus promoters (based on the work of Tempera et al. 2011). (**a**) In latency type I, *oriP*, which functions as an enhancer, associates with the chromatin insulator protein CTCF (*blue hexagonal symbol*) bound at Qp. Qp is on; Cp is off. (**b**) In latency type III, *ori*P interacts with a region upstream of Cp via CTCF (*blue hexagonal symbol*). Qp is off; Cp is on

the composition of the 3D organization of the EBV episome via recruiting additional nuclear factors and CTCF–CTCF interactions (Takacs et al. 2010). We speculate that a potential nuclear protein recruited by CTCF to EBV episomes could be cohesin, a CTCF partner known to mediate cell type-specific looping events that are unrelated to the regulation of promoter activity in lymphoid cells. Our suggestion is based on the observation of Degner et al., who studied the formation of chromatin loops during recombination of immunoglobulin gene segments. They found very similar CTCF-binding patterns throughout the *IgH* locus in different lymphoid cell types displaying very different looping patterns. In contrast, binding of the cohesin subunit Rad21 was extensive at all of the CTCF sites in pro-B cells active in V-DJ recombination but reduced in pre-B cells and thymocytes that do not show such an activity. Thus, cohesin binding to CTCF sites may facilitate multiple loop formation and V-DJ recombination (Degner et al. 2009). Recently Kang et al. described that both CTCF and cohesin were involved in the organization of chromatin loops of the Kaposi's sarcoma-associated herpesvirus (KSHV) genome (Kang et al. 2011). We conclude that the exact function of alternative 3D patterns of EBV chromatin organization remains to be explored.

Table 8.4 Mechanisms of epigenetic dysregulation in host cells carrying latent Epstein–Barr virus genomes

Latency product	Cellular partner	Putative outcome
EBNA1	EBNA1-binding sites	Demethylation, gene *activation*
EBNA2	Histone acetyltransferases	Gene *activation*
EBNA3C (EBNA6)	Prothymosin alpha, p300; histone deacetylases (HDAC1 and 2)	*Modulating* EBNA2-mediated transactivation
EBNA-LP (EBNA5)	Histone deacetylase 4 (HDAC4)	Displacement of HDAC4 from EBNA2-activated promoters, *coactivation*
LMP1	Upregulation of DNMT1,DNMT3A, and DNMT3B via the JNK pathway	Promoter *silencing*
	Bmi-1 (a component of PRC1)	Promoter *silencing* Promoter *activation*
	KDM6B (histone H3K27me3 demethylase)	Promoter *activation*
	Upregulation of microRNA levels via the NF-κB pathway	miR-146a and miR-155 *Modulate* cellular mRNA levels
	Downregulation of KDM3A (histone H3K9me2 demethylase) via upregulation of miR-155	Promoter *silencing*
LMP2A	Upregulation of DNMT1, DNMT3A, and DNMT3B via the NF-κB pathway	Promoter *silencing*
	Downregulation of KDM3A (histone H3K9me2 demethylase) via upregulation of miR-155	Promoter *silencing*

8.2.3 Epigenetic Alterations in Epstein–Barr Virus-Associated Neoplasms: Common Epigenetic Events and Virus-Specific Epigenetic Signatures

EBV-associated neoplastic cells differ from their normal counterparts regarding their epigenome and gene expression pattern (transcriptome). Tumor-specific epigenetic changes regularly occur, independently of the presence or absence of EBV genomes, in malignant tumors belonging to different histological types. Other tumor-specific epigenetic alterations are restricted to a certain histological type only or appear to be unique for EBV-associated neoplasms (EBV-specific epigenetic signatures).

EBV-specific epigenetic signatures may mark the epigenome of the host cell due to the interaction of certain EBV-encoded proteins with the cellular epigenetic regulatory machinery (Takacs et al. 2009, Table 8.4). The nuclear antigen EBNA1 may elicit, in principle, site-specific DNA demethylation at its cellular binding sites, similarly to the demethylation observed at *oriP*, the latent origin of EBV replication (Lin et al. 2000). The transactivator protein EBNA2, associating with cellular

histone acetyltransferases (Wang et al. 2000), can switch on both viral and cellular promoters. EBNA3C (EBNA6) interacts with both histone deacetylases (HDACs) and histone acetyltransferases (Cotter and Robertson 2000; Knight et al. 2003). EBNA-LP (a leader protein, also called EBNA5) may displace HDAC4 from EBNA2-bound promoters (Portal et al. 2006; Ling et al. 2009). The transmembrane protein LMP1 upregulates DNMT1, DNMT3A, and DNMT3B as well as the PcG group protein Bmi-1 resulting in the silencing of cellular promoters (Tsai et al. 2002, 2006; Dutton et al. 2007). It is worthy to note, however, that Bmi-1 also mediates upregulation of certain LMP1 target genes implicated in lymphomagenesis. LMP1 may also activate its target genes by induction of KDM6B, a histone H3K27me3 demethylase which removes the trimethyl mark from lysine 27 of histone H3 and thereby dissociates PRC1 PRCs from their binding sites (Anderton et al. 2011). LMP1 affects cellular mRNA levels by inducing the expression of cellular microRNAs as well (Motsch et al. 2007; Cameron et al. 2008; Gatto et al. 2008; Rahadiani et al. 2008; Anastasiadou et al. 2010; Li et al. 2010). Similarly to LMP1, the transmembrane protein LMP2A may also upregulate DNMT1 transcription resulting in the inactivation of the tumor suppressor gene *PTEN* (*phosphatase and tensin homologue, deleted on chromosome ten*) by promoter methylation (Hino et al. 2009).

8.2.3.1 Burkitt's Lymphoma: Alternative Scenarios and Epigenetic Changes

BL is a high grade aggressive non-Hodgkin lymphoma (NHL) whose tumor cells resemble germinal center centroblasts. The starry-sky appearance on histological slides is due to interspersed macrophages that clear the cellular debris from the highly apoptosis-prone tumor. Sporadic BL (sBL) occurs worldwide, is EBV-infected in about 20% of cases, and mainly affects young adults. In equatorial Africa and New Guinea, BL occurs endemically (eBL) with a 100-fold higher incidence than sporadic of about 5–10 cases/100,000 children per year and is EBV-infected in more than 95% of cases. The geographical distribution of eBL coincides with areas endemic for malaria tropica. Local hotspots of high BL incidence are likely due to additional arbovirus epidemic outbreaks (van den Bosch 2004). BL occurs with intermediate incidences in other countries, e.g., in Algeria, Egypt, or Brazil where children suffer from a relatively high load of parasites other than *Plasmodium falciparum*. In those countries, the higher incidences in comparison to sBL are made up by EBV-positive cases; thus, those are in reality eBLs. BL incidence is also increased among HIV-infected, but not among otherwise severely immune-suppressed patients.

AIDS-related BL (AIDS-BL) is mostly a disease of the early lymphadenopathic phases of HIV-disease when the germinal centers are hyperactive, the CD4+ cell numbers are still within a normal range, and the immune system is still functional. Two thirds of AIDS-BL cases are EBV-negative in western countries, while they are almost entirely EBV-positive in equatorial Africa. BLs regularly carry a translocation between the c-Myc gene and one of the immunoglobulin loci as a most characteristic genetic lesion which seems to occur during the somatic hypermutation or class switch recombination in the germinal centers of the lymph nodes

(Goossens et al. 1998). The chromosomal translocation points of the eBL and sBL subtypes show a different local preference (Magrath et al. 1992). Myc translocation leads to the activation and constitutive overexpression of c-Myc in B cells. The pleiotropic effects of the transcription factor and chromatin modifier c-Myc on cell growth, cell cycle progression, differentiation, and apoptosis through binding to a set of cellular key promoters and the role of c-Myc overexpression for BL pathology have been extensively reviewed (e.g., Hecht and Aster 2000; Levens 2003). Expression profiling of different types of B-cell lymphomas established a molecular profile for sBL. BLs without c-Myc translocation but with an mRNA expression profile characteristic for BL do exist in about 10% of sporadic cases (Staudt and Dave 2005; Hummel et al. 2006; Dave et al. 2006). The three distinct forms of BL, endemic, sporadic, and AIDS-related, seem to arise from slightly different stages of the germinal center (GC) reaction. Therefore, sBL are supposed to originate from early centroblasts, and eBL from late germinal center cells or memory B cells (Bellan et al. 2005). Indeed, expression profiling showed that BL is a unique entity different from other B-cell malignancies and that the three BL subtypes, eBL, sBL, and AIDS-BL, with few differences in gene expression, are a highly homogeneous group which is also closely related to germinal center cells (Piccaluga et al. 2011). Also miRNA expression profiles were highly similar between the different BL subtypes with only few slight differences. Actually, the EBV and HIV status of the respective BLs had no influence on the miRNA expression profiles (Lenze et al. 2011). Even mouse models for EBV-negative and EBV-associated BL did not yield a significant difference in gene expression between both (Bieging et al. 2011). The overall small differences in the mRNA and miRNA expression profiles between the different BL subtypes support the view that they may represent only one biological entity (Lenze et al. 2011) which fits nicely to our molecular model for the origin of eBL (Niller et al. 2003, 2004a).

This BL model does not rely on the viral growth program and the accompanying expression of the major transforming viral protein of lymphoblastoid cells, EBNA2, which is not expressed in the vast majority of BLs (Kelly et al. 2002). The viral growth program, based on expression of EBNA2, seems not to be compatible with the GC reaction (Tobollik et al. 2006). This is consistent with the observation that EBV-infected cells, although physically located in GCs, did normally not participate in GC reactions which yield a clonal expansion of hundreds to thousands of cells (Araujo et al. 1999; Kurth et al. 2003). Only under conditions of immune hyperstimulation, clonal expansions of EBV-infected B cells that did not express EBNA2 were observed in GC reactions (Araujo et al. 1999). Recently, it was confirmed that EBV-infected cells, even when expressing GC markers, did not express EBNA2 and did not expand in a physiological GC reaction (Roughan et al. 2010). Obviously, LMP1 expression in the GC mostly permits only an abortive GC reaction (Uchida et al. 1999). Our model, therefore, avoids unnecessary posits and, despite converse statements on the presumptive nonexistence of a satisfactory molecular model for the origin of eBL (Thorley-Lawson 2004), remains up and standing, whereas the models relying on the EBNA2 transformation as a requirement for eBL development are unconvincing and have not been supported by later data.

We suggested that in the EBV-infected BL precursor cell, the tumorigenic and proapoptotic c-Myc protein binds to the unique Myc-binding site (Niller et al. 2004c) in the locus control region (LCR) of the viral genome. Myc binding then results both in the constitutive expression of the antiapoptotic EBER genes and supports the attachment of the viral genome to the nuclear matrix. Thereby, the viral genome has a higher likelihood to stick around in the nucleus and additional antiapoptotic viral genes of the restricted viral latency programs, e.g., EBNA1, LMP1, or LMP2A, to be expressed. Thus, the balance between apoptosis and antiapoptosis becomes permanently shifted in favor of cell survival. This allows the highly expressed c-Myc to exert its oncogenic potential and to drive lymphomagenesis in the EBV-infected B cell that happened to undergo an accidental c-Myc translocation on its way through the germinal center reaction (Niller et al. 2004b; Rossi and Bonetti 2004). This model is somewhat analogous to the pancreatic tumor model described by Pelengaris et al. (2002). However, we would like to note that from the beginning our model did not only rely on the antiapoptotic effects of the EBER RNAs but included the matrix attachment and locus control functions of c-Myc on the viral genome (Niller et al. 2003).

Tumor-specific promoter hypermethylation in BLs frequently inactivates tumor suppressor genes that are silenced, in addition to BLs, in a wide variety of carcinomas and lymphomas as well. It is also noteworthy that some of these changes could be observed only in a fraction of the EBV-positive BLs studied. As a matter of fact there is no expression of either LMP1 or LMP2A, the EBV-encoded proteins capable to induce de novo methylation, in BL cells. Thus, one may wonder how the apparently EBV-specific epigenetic signatures, e.g., hypermethylation of the activated B-cell factor (*ABF1*) gene, involved in the survival or activation of B cells, or de novo methylation of *HOXB13, CALCA, NEFL*, and *PROK2* genes marked by PRC in embryonic stem cells, are generated in BLs (Ushmorov et al. 2008; Martin-Subero et al. 2009). We speculate that EBV latency products that have not been implicated yet in the induction of cellular promoter hypermethylation may play such an unsuspected role: EBNA1, EBER1, EBER2, BART transcripts, BART encoded proteins, and microRNAs processed from BART transcripts are potential candidates. Alternatively, the observed epigenetic signatures attributed to the presence of EBV genomes could be generated by epigenetic remodeling during lymphomagenesis, independently of the putative activities of EBV latency products. In this context, one may consider that ABF1 is a transcriptional repressor interfering with the activating functions of E2A proteins that regulate early B-cell development (Massari et al. 1998). Accordingly, its expression contributes to the non-B-cell phenotype in HL, whereas the silencing of the ABF1 promoter by methylation occurs not only in BL cells carrying latent EBV genomes but also in other lymphomas of B-cell origin, including diffuse large B-cell lymphomas (DLBCLs) and follicular lymphomas that are less frequently associated with EBV (Ushmorov et al. 2008). Similarly, the stem cell chromatin pattern observed in EBV-positive BLs, i.e., the presence of a set of de novo methylated genes (*HOXB13, CALCA, NEFL, PROK2*) that are repressed by PRC2 in embryonic stem cells, could also be observed in DLBCLs in spite of the difference of BLs and DLBCLs regarding morphology, genetic background, and transcriptional pattern (Martin-Subero et al. 2009).

Based on these observations, Martin-Subero et al. speculated that BLs and DLBCLs either originate from cells with stem cell features or acquire such features, i.e., a stem cell-like chromatin pattern, during lymphomagenesis by remodeling their epigenotypes in a similar manner (Martin-Subero et al. 2009).

8.2.3.2 Hodgkin's Lymphoma

Like BL, classical Hodgkin's lymphoma (cHL) is a lymphoma derived from the germinal center reaction. The mononuclear Hodgkin and polynuclear Reed–Sternberg cells which comprise only about 1–2% of the total Hodgkin tumor mass are crippled GC B cells that escaped imminent apoptosis (Kuppers et al. 2002). However, cHL is an atypical B-cell lymphoma which has lost its B-cell identity (Kuppers 2009). Classical HL is grouped into four subtypes: mixed cellularity, lymphocyte depleted, nodular sclerosis, and lymphocyte predominant that are EBV-positive to varying degrees (Herbst et al. 1991; Pallesen et al. 1991, reviewed by Niller et al. 2007; Hjalgrim and Engels 2008). In Europe and the USA, the overall rate of EBV-infected tumors is between 40 and 50%, whereas in immunocompromised patients and in developing countries, the EBV-positive rate is much higher (Jarrett 2003; Hjalgrim et al. 2003; Dinand and Arya 2006; Grogg et al. 2007; Hjalgrim and Engels 2008).

Although Hodgkin and Reed–Sternberg (HRS) tumor cells are derived from B cells and carry functional rearrangements of immunoglobulin gene segments, they do not produce immunoglobulins. This can be attributed partly to mutations, partly to epigenetic alterations, and predominantly hypermethylation that suppresses the IgH promoter as well as a set of genes coding for B cell-specific transcription factors. Silencing of the genes for transcription factors OCT2, BOB.1, and PU1 as well as other B cell-specific genes including *CD19, CD20, CD78B, SYK*, *BCMA*, and *LCK* is attributed to the demethylation and expression of regulator genes *ABF1* and *ID2* suppressing the B cell-specific gene expression program in HRS cells. Other nuclear proteins, like GATA-3, a T and NK cell-specific transcription factor that is ectopically expressed in HL, the transcriptional repressor EAR3, the transcription factor Nrf3, and the tyrosine kinase FER, are also upregulated in HRS cells (Kuppers et al. 2003). It is interesting to note that a set of genes hypermethylated in human embryonic stem cells and CD34+ hematopoietic stem and progenitor cells were found to be demethylated during hematopoietic differentiation in a cell type-specific fashion (Calvanese et al. 2012). These included *CD19, RUNX3, CD79B*, and *TCL1* that are among the genes hypermethylated in HRS cells. Based on these observations, Calvanese et al. suggested that an unmethylated promoter landscape is established during successive stages of hematopoietic development and lymphoid differentiation. This process is disturbed, however, in neoplastic cells (Calvanese et al. 2012). Thus, the essence of epigenetic dysregulation in HL could be a defective demethylation process, rather than an anomalous de novo hypermethylation.

Although defective demethylation could certainly shape their epigenome, de novo DNA methylation induced by viral proteins is also a plausible mechanism for epigenetic dysregulation in EBV-positive HRS cells. LMP1, a transmembrane

protein frequently expressed both in EBV-positive HLs and EBV-associated NPCs, is capable to upregulate the activity of DNMT1, DNMT3A, and DNMT3B, as well as the expression of the PcG protein Bmi-1 (see Sect. 2.3). Upregulation of DNMT1 by LMP1 involves the c-Jun NH2-terminal kinase/activator protein-1 (JNK/AP-1) pathway and may contribute, in principle, to CpG methylation-mediated silencing of a set of cellular promoters. In germinal center B cells infected by EBV in vitro, DNMT3A was upregulated and bound to the W promoter, where EBNA transcripts are initiated early after infection (Leonard et al. 2011). In parallel, there were non-random methylation changes at cellular genes consistent with an "instructive pattern" of methylation. DNMT3B and DNMT1 were downregulated, however, both in infected germinal center B cells and in HL cell lines (Leonard et al. 2011).

LMP1 is known to activate the NF-κB pathway as well, and this very same pathway mediates induction of the PcG protein Bmi-1. Based on this observation, Dutton et al. suggested that LMP1 may contribute to the loss of B-cell identity in EBV-positive HLs by increasing the level of Bmi-1 that inhibits the expression of a series of B-cell markers, including CD21/MS4A1, BLK, and LY9 (Dutton et al. 2007). In EBV-negative HLs, other activators of the NF-κB pathway may take over the function of LMP1. It is important to note that Bmi-1 is involved in gene activation as well, upregulating the expression of *STAT1*, *c-MET*, and *HK2*. These genes, coding for signaling molecules and hexokinase, an enzyme maintaining the high glycolytic activity of neoplastic cells, are known as the transcriptional targets of LMP1. LMP1 may upregulate its target genes in HLs by the induction of KDM6B, a H3K27me3 demethylase that removes the trimethyl mark from lysine 27 of histone H3 and thereby derepresses genes silenced by PRC1. Such a mechanism may activate a set of LMP1 target genes (*CCR7*, *NOTCH2NL*, *IER3*, *NOTCH2*, and *CD58*) in germinal center B cells, the presumptive progenitors of HRS tumor cells (Anderton et al. 2011).

LMP2A, a transmembrane protein capable to activate DNMT1 transcription in gastric carcinoma cells (Hino et al. 2009), is also expressed in EBV-positive HLs. Thus, in principle, LMP2A may also be involved in the silencing of cellular promoters in HRS tumor cells. In summary, latent EBV proteins may contribute to gene silencing events in HL by upregulating DNMTs. In addition, the LMP1-induced PcG protein Bmi-1 may inactivate cellular promoters either directly or, perhaps, by recruiting DNMTs. Bmi-1 may also mediate the activation of LMP1 target genes implicated in lymphomagenesis, in concert with the LMP1-induced histone demethylase KDM6B.

8.2.3.3 Nasopharyngeal Carcinoma

NPC is a lymphoepithelial tumor derived from the epithelium of the nasopharyngeal surface, in particular the fossa of Rosenmüller. Practically all anaplastic NPCs studied carry latent EBV episomes. This undifferentiated NPC subtype is highly endemic in South-East Asia. In some areas of South-East China, NPC has the highest incidence of all tumors among men. In Tunisians and Alaskan Inuit, NPC is endemic with an intermediate incidence. Incidences in Hong Kong, however, are receding due to changing eating habits and an improved early detection. Cantonese in the

Guangzhou area seem to carry a higher genetic risk for developing NPC than North Chinese in addition to the increased environmental risk due to tumor-promoting substances in local teas and fish (reviewed by Niller et al. 2007). Terminal repeat analysis of the circular viral genomes indicated that NPCs arose by monoclonal proliferation of tumor cells (Raab-Traub and Flynn 1986). This observation is consistent with a scenario envisaging EBV infection of NPC cells or their precursors already at an initial phase of carcinogenesis. The EBV oncoprotein LMP1 is frequently expressed in NPCs, and its ability to upregulate cellular DNMTs and thereby promote the formation of a transcriptional repression complex on the E-cadherin promoter resulting in hypermethylation and silencing of the promoter may directly affect tumor progression. It was observed that the frequency of *E-cadherin* (also called *CDH1*) promoter hypermethylation increased with advanced disease stage and in lymph node metastases of NPCs carrying EBV genomes (Zheng et al. 1999; Krishna et al. 2005; Niemhom et al. 2008). This indicates that LMP1-mediated silencing of a cellular gene may contribute to the progression of NPC.

By activating the NF-κB pathway, LMP1 may affect the levels of certain cellular microRNAs (miR-146a, miR-155) and thereby modulate, in a subtle way, cellular mRNA levels (Motsch et al. 2007; Cameron et al. 2008; Gatto et al. 2008; Rahadiani et al. 2008; Anastasiadou et al. 2010). NPCs express high levels of viral microRNAs as well, processed from the BART transcripts. Three of the EBV-encoded microRNAs target the LMP1 mRNA and may thereby downregulate the level of LMP1 protein in NPC cells (Lo et al. 2007; Swaminathan 2008).

A series of tumor suppressor and candidate tumor suppressor genes are regularly inactivated by promoter hypermethylation in NPCs. Their exact role in the initiation and progression of NPC remains to be established. Inactivation of the genes coding for retinoic receptor β2 (RARβ2), cellular retinol-binding proteins 1 and 4 (CRBP1, CRBP4), and retinoic acid receptor responder (RARRES/TIG1) may explain the failure of 13-*cis*-retinoic acid therapy of NPC (Kwong et al. 2002, 2005a, b; Sriuranpong et al. 2004).

8.2.3.4 EBV-Associated Gastric Carcinoma

Gastric carcinoma is among the most frequent cancers and most frequent causes of cancer-associated death worldwide, however, with large geographical differences. Although the majority of gastric carcinomas is associated with *H. pylori* infection, a subgroup of about 10% of gastric adenocarcinomas developing mostly in the upper stomach carries latent EBV genomes in the tumor cells. Adenocarcinomas are EBV-positive in about 10% of cases, the rare lymphoepithelioma subtype in more than 80% of cases. The histology of the lymphoepitheliomas resembles that of NPC. Gastric-stump carcinoma carries an intermediate frequency of EBV-positive tumors. Beyond the mucosal or in situ stage, tumors are monoclonal (reviewed by Niller et al. 2009). The methylation pattern of EBV-associated gastric carcinomas was reviewed recently (Fukayama et al. 2008; Niller et al. 2009). Most of the hypermethylated sequences were localized to so-called CpG islands (CGIs). In normal cells, CGIs that

contain a higher than average level of CpG dinucleotides are typically unmethylated, and a significant fraction of cellular promoters are located on CGIs. Such CGI-associated promoters are frequently methylated, however, in certain neoplasms (CpG island methylator phenotype, CIMP). EBV-associated gastric carcinomas belong to this category, and it was observed that successive stages of neoplastic development were associated with a progressively increasing level of CGI methylation. A series of tumor suppressor genes were found to be methylated in EBV-associated gastric carcinomas. In contrast, DNA repair genes (*hMLH1* and *hMSH2*) were not inactivated by CpG methylation in EBV-positive NPCs. A fine analysis of methylation patterns showed that although the p16INK4A and p14ARF promoters were highly methylated in both EBV-negative and EBV-positive gastric carcinomas, they could be distinguished based on the methylation profiles of individual CpG motifs (Sakuma et al. 2004). How these unique methylation profiles were generated remains to be analyzed. In addition, a high level of p73 methylation could be detected in EBV-positive gastric carcinomas only (Ushiku et al. 2007), and methylation of a homeobox gene, *HOXA10*, was also associated with EBV-positive carcinomas of the stomach (Kang et al. 2008). These observations indicate that EBV-specific epigenetic signatures are left on the host cell epigenome in EBV-associated gastric carcinomas and such epigenetic marks may play a role in epigenetic reprogramming of gene expression in gastric carcinoma cells.

Although LMP1, a latent EBV protein capable to upregulate maintenance and de novo DNMT levels, could not be detected in gastric carcinomas, a fraction of EBV-positive gastric cancers express LMP2A that may contribute to epigenetic remodeling of the host cell epigenome via the activation of DNMT1 transcription (Hino et al. 2009, Table 8.4). How the upregulated DNMT is targeted to selected cellular promoters remains to be established.

8.2.3.5 Posttransplant Lymphoproliferative Disorder

A high EBV load and increased number of EBV-infected B cells could be observed in organ and bone marrow transplant patients before the onset of posttransplant lymphoproliferative disorder (PTLD) (Stevens et al. 2001; Muti et al. 2003; Baudouin et al. 2004; Davis et al. 2004). The overall PTLD frequency after allogeneic transplants is about 1%, but strongly depends on the specific organ, type and duration of immune suppression, patient age, and the EBV serostatus of donor organ and recipient (Gottschalk et al. 2005; Kamdar et al. 2011). Early lesions correspond to polyclonal proliferation of B cells carrying latent EBV genomes which express all latency genes (latency type III), similarly to the B-cell proliferations observed in IM patients (Brink et al. 1997; Tanner and Alfieri 2001, reviewed by Niller et al. 2007). In contrast to IM, where an efficient immune response curbs the B-cell proliferation in most cases, there is an uncontrolled proliferation of EBV-infected B cells in transplant patients, due to the immunosuppressive regimen. The clonality of the early-onset tumors may change towards monoclonality due to clonal evolution: More malignant late-onset PTLD may already begin with monoclonality and includes more monomorphic than polymorphic PTLD (Kremer et al. 2011). Late-onset PTLD may also include BL, large B-cell lymphoma, and HL. Accordingly,

latency types I as in BL and II as in HL also occur among EBV-positive PTLDs (Brauninger et al. 2003; Timms et al. 2003). The pathogenetic mechanisms of early-onset PTLD and late-onset PTLD are different, as the latter one is due to a composite of both immune stimulation through the transplanted organ and therapeutic immune suppression (reviewed by Niller et al. 2004b).

Rossi et al. observed that *DAP-K*, a gene encoding a serine–threonine protein kinase involved in apoptosis induction, is frequently inactivated by promoter hypermethylation in monomorphic PTLD (Rossi et al. 2003). Downregulation of DAP-K may block the IFNγ-, TNFα-, and FasL-triggered apoptosis pathway and may thereby provide a selective advantage for proliferating cells carrying hypermethylated *DAP-K* alleles. In addition to *DAP-K*, the promoter for *MGMT*, a gene encoding O6-methylguanine DNMT, was also frequently inactivated by CpG methylation in monomorphic PTLD. MGMT removes DNA adducts formed by alkylating agents, and its inactivation may result in the accumulation of mutations that may facilitate lymphomagenesis or progression of the early PTLD lesions. Hypermethylation of the candidate tumor suppressor gene p73 was observed less frequently in PTLD (Rossi et al. 2003). The contribution of latent EBV proteins capable to upregulate DNMTs (LMP1, LMP2A) in PTLD-associated hypermethylation of cellular promoters remains to be clarified.

8.3 Epigenetic Changes in Neoplasms Associated with Kaposi's Sarcoma-Associated Herpesvirus

KSHV, also called human herpesvirus-8 (HHV-8), is a human gammaherpesvirus that was discovered first as a herpesvirus-like DNA sequence in AIDS-associated Kaposi's sarcoma, a malignant tumor of endothelial origin (Chang et al. 1994). The cells of two B-lymphocyte disorders, primary effusion lymphoma (PEL, also called body cavity-based lymphoma, BCBL) and multicentric Castleman's disease (MCD), also carry KSHV genomes.

8.3.1 Kaposi's Sarcoma-Associated Herpesvirus: Basic Facts and Epigenetic Landscape of the Latent Viral Genomes

After primary infection and initial productive (lytic) replication, KSHV appears to establish lifelong latency in B cells, similarly to EBV. The linear double-stranded DNA (dsDNA) genome packaged into the virions ends in terminal repetitions (TRs) that fuse with each other in latently infected cells. Thus, latent KSHV genomes persist as circular episomes and coreplicate with the cellular DNA, like the EBV episomes (reviewed by Pantry and Medveczky 2009; Tempera and Lieberman 2010).

A further similarity between the two human gammaherpesviruses is the association of the latency-associated nuclear antigen (LANA) of KSHV and EBNA1 of EBV

with their latent origins of viral DNA replication that are located at the terminal repeats of KSHV and at *oriP* of EBV, respectively (reviewed by Tempera and Lieberman 2010). However, the epigenetic consequences of these protein–DNA interactions are somewhat different. LANA recruits HP1 (heterochromatin-associated protein 1) and another key component of heterochromatin formation, SUV39H1, one of the histone lysine methyltransferases acting on lysine 9 of histone H3, to the TRs (Lim et al. 2003; Sakakibara et al. 2004). Although these proteins are regularly associated with a closed chromatin structure, the TRs of KSHV are not associated with an elevated level of H3K9me3; instead, they are rich in activating histone modifications (Gunther and Grundhoff 2010). Stedman et al. also found that a combination of origin binding factors, bromodomain proteins, and histone acetyltransferases created a region of histone H3 hyperacetylation, restricted to sequences adjacent to the LANA-binding sites, at the latent origin of replication of the KSHV episomes (Stedman et al. 2004). This hyperacetylated region was flanked with hypoacetylated areas.

A major difference between the replication origins of KSHV and EBV is that EBNA1-bound *oriP* can function as a long-range transcriptional enhancer, a property not shared with the TRs of KSHV. *OriP* is enriched in H3K4me2, an activating histone mark, although in the late G1 phase of cell cycle its dyad symmetry element (DS) undergoes nucleosome remodeling in parallel with deacetylation of the associated histone H3 (Zhou et al. 2005).

According to a recent study, a most characteristic epigenetic feature of latent KSHV episomes appeared to be the deposition of both repressive (H3K27me3) and activating (H3K4me3) histone marks across the genome (Gunther and Grundhoff 2010). It is remarkable, however, that the major latency promoter located upstream of ORF73 showed very little or no H3K27 trimethylation both in BCBL1, a body cavity-based lymphoma cell line and a cell line of endothelial origin that carried strictly latent KSHV genomes. The overlapping region between nucleotides 127301 and 128901 of the KSHV genome was found to be hypomethylated in the same cell cultures and in additional lymphoma cell lines, in spite of the fact that in case of BCBL1 other regions of the genome were highly methylated. Others described binding of the histone methyltransferase EZH2 to the regions marked with H3K27me3 (Toth et al. 2010). In contrast, repressive histone marks could not be consistently detected in association with latent EBV genomes (Chau and Lieberman 2004; Day et al. 2007). Recently Kang et al. described that latent and lytic KSHV genomes are organized into chromatin loops by CTCF- and cohesin-mediated interactions. They argued that the alternative conformations of the genome may control latent and lytic gene expression programs (Kang et al. 2011).

8.3.2 Modulation of the Host Cell Epigenotype by the KSHV Nuclear Antigen LANA

As described above, interaction of LANA with the latent origin of KSHV replication results in the recruitment of nuclear proteins potentially involved in the formation of

heterochromatin, although, paradoxically, the expected repressive chromatin marks were not detected at the TRs in genome-wide studies. However, a repressive chromatin structure was observed, indeed, at the LANA-binding sites of the cellular genome. LANA recruits DNMT3A, but also DNMT1 and DNMT3B to selected cellular promoters which results in promoter silencing via CpG methylation (Shamay et al. 2006). In addition, LANA interacts with the mSIN3A transcriptional corepressor complex that attracts histone deacetylases to the chromatin (Krithivas et al. 2000). Association of LANA with HP1 was also demonstrated and may target LANA and the LANA-associated KSHV episomes to heterochromatic regions within the nucleus (Lim et al. 2003; Sakakibara et al. 2004). In addition, LANA associates with MeCP2 (Matsumura et al. 2010). MeCP2, a multifunctional protein initially implicated in transcriptional silencing of methylated promoters (Nan et al. 1998a), is involved both in gene repression and gene activation (reviewed by Hansen et al. 2010; see also Chap. 3). MeCP2 facilitated both the repressor and the transactivator function of LANA, depending on the promoter analyzed (Matsumura et al. 2010). Since MeCP2 bound both methylated and unmethylated regions of the cellular genome (Yasui et al. 2007; Chahrour et al. 2008), the ability of LANA to establish an interaction with MeCP2 may direct LANA to diverse areas of the genome, resulting in gene repression or activation in a context-dependent manner.

Stuber et al. observed that in mouse cells, exogenously expressed LANA reorganized the chromatin via interaction with MeCP2 and the histone methyltransferase SUV39H1 (Stuber et al. 2007). They speculated that LANA-induced changes in the positioning of chromosomal domains in interphase nuclei may contribute to the chromosomal rearrangements and translocations observed in BCBL/PEL cell lines. They further suggested that LANA and the anchored viral episome fixed to the borders of heterochromatin may act as an epigenetic modifier, generating locally altered chromatin states. Such altered chromatin states may occur at random positions and may target genes involved in growth control by chance. Once established, however, the altered epigenetic states may be propagated during cell divisions and contribute to tumorigenesis (Stuber et al. 2007). LANA binds, induces, and relocates to nuclear heterochromatic regions Brd2/RING3, a bromodomain containing chromatin-binding protein involved in E2F-mediated activation of cell cycle regulatory genes (Platt et al. 2002; Mattsson et al. 2002; Viejo-Borbolla et al. 2005). Recent data that demonstrated a specific interaction of Brd2/RING3 with both mono- (H4K12ac) and diacetylated (H4K5ac/K12ac) histone H4 molecules (Umehara et al. 2010) are in accordance with the idea of Mattsson et al., who speculated that although the KSHV episomes are anchored to the host heterochromatin, heterochromatinization is possibly inhibited in their immediate neighborhood, due to the action of LANA and Brd2/RING3 (Mattsson et al. 2002). These LANA-induced epigenetic alterations may contribute to the gene silencing and activation events observed in cells carrying latent KSHV genomes. In endothelial cells, LANA-silenced cellular genes include *H-cadherin* (*CDH13*), *CCND2*, *LDHB*, *FOXG1B/FKHL1*, and *CREG*, whereas *p16INK4A* and *MGMT* were found to be silenced in PEL cell lines (Carbone et al. 2000; Platt et al. 2002; Shamay et al. 2006). The genes activated by LANA are mostly interferon-inducible genes encoding MxA, a protein blocking viral RNA-dependent

RNA polymerases, and transcription factors like Staf-50 (stimulated trans-acting factor of 50 kDa). LANA also upregulated the mRNAs of less well-characterized proteins (IFI 9-27, IFI 6-16, Renne et al. 2001). The role of these products in the pathobiology of KSHV infection remains to be elucidated.

8.4 Human T-Lymphotropic Virus Type I: "Hit and Run" Tumorigenesis Mediated by CpG Island Methylation?

Human T-lymphotropic virus type I (HTLV-I) infection is associated with adult T-cell lymphoma and leukemia. Similarly to other retroviruses, the RNA genome of HTLV-I is copied to dsDNA by the viral enzyme reverse transcriptase, and the DNA copy integrates into the cellular genome. The integrated HTLV-I genome (provirus) is transcribed by the cellular RNA polymerase II enzyme. Both genome-length and processed viral transcripts are transported to the cytoplasm, where the translated viral proteins assemble into virions engulfing the viral genomes and leave cells by budding through the cell membrane. Tax, the transactivator protein of HTLV-I was implicated in lymphomagenesis. Tax expression itself is downregulated, however, in a significant fraction of the tumors due to deletions or epigenetic silencing. Tax can activate a set of cellular genes, but it was also observed that this oncoprotein contributes to the silencing of *Shp1*, a gene coding for the Src homology containing protein tyrosine phosphatase. Tax-mediated silencing was attributed to the displacement of transcription factors by Tax from the *Shp1* promoter, followed by promoter hypermethylation (Nakase et al. 2009). Based on these observations, Niller et al. suggested that Tax may induce an epigenetic change at an initial phase of leukemogenesis and its presence may be dispensable, thereafter, during tumor progression (a "hit and run" scenario, Niller et al. 2011).

8.5 Epigenetic Alterations in Hepatitis B Virus-Associated Hepatocellular Carcinoma

Hepatitis B virus (HBV) is the human pathogenic representative among the *Orthohepadnavirus* genus. This virus group is characterized by a partially dsDNA genome with a DNA polymerase that still includes a reverse transcriptase function. This indicates that the replication mechanisms of hepadnaviruses are phylogenetically in between those of retroviruses and DNA viruses. HBV infects more than two billion people worldwide, and chronic HBV infection frequently results in liver cirrhosis, a condition facilitating the development of hepatocellular carcinoma (HCC) in a multistep process (reviewed by Lupberger and Hildt 2007; Tischoff and Tannapfel 2008).

8.5.1 HBV: Basic Facts and the Methylome of the Viral Genome

HBV infects the liver, thereby inducing a strong immune response against the infected cells which leads, after an incubation time of 2–6 months, to acute hepatitis in about 35% of cases. Mostly however, the immune response is not strong enough to produce icteric or other clinical symptoms. Contrary, an excessive immune response may lead to acute liver failure in less than 1% of cases. An immature immune system, like in newborns, or an insufficient immune response, in 5–10% of adults, is not able to resolve the inflammation, and chronic hepatitis may result. Infectious virus is shed from the liver to the blood and parenterally or sexually transmitted. Although integration into the cellular DNA is not a necessary step during HBV replication, most of the HBV-associated HCCs carry integrated HBV genomes (reviewed by Gatza et al. 2005; Lupberger and Hildt 2007).

Fernandez et al. constructed high-resolution CpG methylation maps of the HBV genome using samples derived from different stages of liver tumorigenesis including chronic active hepatitis, hepatic cirrhosis, and primary hepatocarcinoma. They also studied hepatic cancer cell lines. They found that the HBV genome was almost completely unmethylated in chronic active hepatitis and cirrhosis, but methylated regions could be observed in primary hepatocarcinomas and in in vitro cultivated cell lines derived from liver cancer. Methylation of the coding sequences of core (C) and surface (S) viral proteins was correlated with the lack of expression, whereas the coding sequence of the putative HBV oncoprotein, HB-X (also called pX), was unmethylated even in genomes highly methylated at other regions. Genetic changes, i.e., deletions, of the HBV genome were also recorded, more frequently in chronic active hepatitis and hepatic cirrhosis than in liver tumors (Fernandez et al. 2009).

Kaur et al. reported that three CGIs of the HBV genome were unmethylated in liver samples of chronic hepatitis patients. CGI 1, spanning the ATG start site of the surface antigen gene, was methylated, however, in 18% of cirrhotic liver samples and 30% of liver carcinomas. They argued that in HBV-infected normal hepatocytes, unlike in liver carcinoma cells, DNA methylation does not play a role in the chronic silencing of HBV surface antigen genes (Kaur et al. 2010). Thus, other epigenetic mechanisms may be responsible for the silencing of surface antigen genes in patients tested HBsAg-negative in spite of the occult HBV infection of the liver.

8.5.2 Remodeling of the Host Cell Epigenome in HBV-Associated Hepatocarcinoma: Regional Hypermethylation Versus Global Hypomethylation

Regional DNA hypermethylation that results in silencing of key cellular genes involved in cell proliferation, apoptosis, DNA repair, cell adhesion, and invasion occurs frequently in neoplastic cells (Baylin and Herman 2000). Another important phenomenon observed in neoplastic cells is a global hypomethylation of the genome,

attributed mainly to a decreased methylation of repetitive sequences (reviewed by Ehrlich 2000; Hansen et al. 2011).

The viral oncoprotein HB-X (also called pX), a pleiotropic regulator expressed in hepatocarcinoma cells, not only modulates multiple signaling pathways but also interacts with the transcription factors TBP, CREB, and ATF2 directly, and activates thereby a diverse set of cellular genes coding for nuclear proteins (c-fms, c-myc), MHC class I and II proteins, interferon-β, metallothionein, and β-actin. In addition, HB-X appears to be unique among viral oncoproteins because it differentially affects the level of cellular DNA methyltransferases: HB-X upregulates DNMT1 and DNMT3A but downregulates DNMT3B. Upregulation of DNMT1 and DNMT3A1 results in local hypermethylation of selected tumor suppressor genes, including *E-cadherin* and *RASSF1A*, which occurs in parallel with a global hypomethylation of the genome, as a consequence of the HB-X-mediated downregulation of DNMT3B involved in methylation of satellite 2 repeat sequences (Park et al. 2007).

Saito et al. observed that DNMT3B4, a protein translated from a splice variant of the DNMT3B transcript, is overexpressed in chronic hepatitis, cirrhotic liver, and HCC (Saito et al. 2002). They also observed that increased levels of DNMT3B4, compared to DNMT3B3 translated from the major splice variant, correlated with hypomethylation of pericentromeric satellite regions, possibly due to an impaired DNMT activity of the DNMT3B4 variant protein that lacks the conserved methyltransferase motifs IX and X. Transfection of DNMT3B4 cDNA into 293 cells, an adenovirus DNA-transformed human embryonic kidney cell line, induced DNA demethylation on satellite 2 sequences in pericentromeric heterochromatin DNA and resulted in an increased growth rate of the cells. Saito et al. speculated that DNA hypomethylation at these regions, especially adjacent to the centromeres of chromosomes 1 and 16, abundant in satellite 2 sequences, may lead to chromosomal instability already in the precancerous stages of hepatocarcinogenesis. In addition, hypomethylation of certain CGI genes located to heterochromatic regions may result in aberrant expression of cancer-related genes, facilitating hepatocarcinogenesis (Saito et al. 2002). How the HB-X-mediated downregulation of DNMT3B is related to the overexpression of DNMT3B4 remains to be clarified.

The relationship of HB-X to SALL3 (sal-like 3), a protein inhibiting the association of the de novo DNMT3A to chromatin (Shikauchi et al. 2009), also remains to be established, especially with regard to the DNA methylation-mediated silencing of *SALL3* in HCCs (Shikauchi et al. 2009). One may wonder whether HB-X could recruit DNMTs to the *SALL3* promoter. Lambert et al. detected tumor-specific hypermethylation of specific genes (*GSTP1, RASSF1A, CHRNA3, DOK1*) in hepatocarcinomas compared to control cirrhotic or normal liver tissues. There was a correlation between hypomethylation of *MGMT* (O6-methylguanine DNMT) which is involved in DNA repair, alcohol intake as well as hypermethylation of *GSTP1* (glutathione S-transferase P1) that is inactivating electrophilic carcinogens, and HBV infection (Lambert et al. 2011). *GSTP1* hypermethylation was observed, however, in other carcinomas (prostate cancer, breast cancer, cholangiocarcinoma) as well (reviewed by Tischoff and Tannapfel 2008). In addition, Zhang et al. found a significant correlation between *GSTP1* hypermethylation and aflatoxin B1-mediated

hepatocarcinogenesis (Zhang et al. 2005). One may speculate, however, that viral and chemical pathways of hepatocarcinogenesis may share certain targets, because Su et al. demonstrated an increased *GSTP1* methylation in HBV-positive hepatocarcinomas, but not in HBV-negative tumors. They also observed that *GSTP1* methylation was higher in cirrhotic versus noncirrhotic tissues (Su et al. 2007).

The promoter region of *RASSF1A*, a multifunctional tumor suppressor gene, was intensively methylated in 95% of HBV-associated HCCs (Zhong et al. 2003). Although heterogenous CpG methylation was found at a lower level also in 70% of the analyzed nontumorous tissues, hypermethylation of the *RASSF1A* promoter was diagnostic for the neoplastic samples in this study. *RASSF1A* is frequently inactivated by promoter methylation in other primary tumors as well, including lung carcinoma, breast carcinoma, prostate carcinoma, neuroblastoma, medulloblastoma, NPC, and HL (reviewed by Donninger et al. 2007). Since the RAS association domain family 1 isoform A (RASSF1A) protein, in addition to modulating multiple apoptotic and cell cycle checkpoint pathways, stabilizes the microtubules (Liu et al. 2003), its downregulation may result in genomic instability during hepatocarcinogenesis. Laurent-Puig and Zucman-Rossi defined two pathways of hepatocarcinogenesis according to the presence or absence of chromosomal instability. HBV-positive tumors fell into the chromosome instable category (Laurent-Puig and Zucman-Rossi 2006). In accordance with the above proposal, all HBV-positive HCCs showed chromosomal instability (CIN) and associated with a high level of CpG methylation of selected CGIs including the *RASSF1A* promoter in a study by Katoh et al. In contrast, all tumors with a methylator phenotype but without CIN were associated with hepatitis C virus (HCV) infection (Katoh et al. 2006). We speculate that the epigenetic downregulation of RASSF1A expression may contribute to the chromosomal instability in HBV-associated liver carcinomas.

In addition to HCCs, *CHRNA3* and *DOK1* were also found to be frequently hypermethylated in gastric carcinoma (Balassiano et al. 2011). *CHRNA3* codes for the α3 subunit of neuronal nicotinic acetylcholine receptors (Bonati et al. 2000), and its polymorphism was found to be associated with an increased risk for squamous carcinoma of the lung (Kohno et al. 2011). The significance of CHRNA downregulation in hepatocarcinogenesis is unknown at present. *DOK1* is a putative tumor suppressor gene that is inactivated by hypermethylation in 93% of head and neck cancer, 81% of lung cancer, and 64% of Burkitt's lymphoma samples (Saulnier et al. 2011). Dok1, a docking protein that is the common substrate for activated protein-tyrosine kinases, functions as an adapter molecule anchoring these enzymes to subcellular structures (Shi et al. 2004). Dok1 appears to oppose oncogenic tyrosine kinase-mediated cell transformation (Janas and Van Aelst 2011). Thus, in principle, downregulation of Dok1 may facilitate hepatocarcinogenesis.

HBV-related HCC develops through distinct stages, and epigenetic alterations may appear already in the preneoplastic lesions. Hypermethylation of the *P16INK4A* tumor suppressor gene was observed in 62% of cirrhotic nodules that are putative preneoplastic lesions and in 70% of dysplastic nodules that surrounded HBV-positive HCC lesions (Shim et al. 2003). Um et al. described that the tumor suppressor genes *APC* (adenomatous polyposis coli) and *RASSF1A*, as well as *SOCS-1*

(suppressor of cytokine signaling-1), which is a negative regulator of the JAK/STAT pathway, were methylated in a fraction of cirrhotic nodules, but the methylation levels of *APC* and *RASSF1* increased further in low-grade dysplastic nodules. *SOCS-1* methylation gradually increased during multistep carcinogenesis, peaked in early HCC, and decreased in progressed liver carcinomas (Um et al. 2011).

Feng et al. compared the epigenetic profiles of HBV-associated and HCV-associated HCCs. They found that *HOXA,* a gene coding for a transcription factor implicated in the regulation of hematopoiesis, and *SFRP1* (secreted frizzled-related protein 1) as well as *RASSF1* were preferentially methylated in HBV-positive HCCs (Feng et al. 2010). SFRP1, a putative tumor suppressor protein that acts as a Wnt signaling modulator, is frequently inactivated in gastric carcinoma and esophageal carcinoma (Kinoshita et al. 2011; Meng et al. 2011). It inhibits Wnt signaling and thereby angiogenesis and tumor growth in HCC (Hu et al. 2009). In HCV-associated HCCs, *CDKN2A* (*p16INK4A* and *p14ARF*) was more frequently methylated (57%) than in HBV-positive tumors (17%) (Feng et al. 2010). These data suggest that unique, HBV- or HCV-specific epigenetic signatures may mark the DNA of virus-associated HCCs.

8.6 Epigenetic Dysregulation of the Host Cell Genome in Hepatitis C Virus-Associated Hepatocellular Carcinoma

HCV belongs to the *Hepacivirus* genus of the *Flaviviridae* virus family. HCV causes inapparent infection in the majority of cases. Symptoms develop in approximately 10–15% of acute infections, but 75–80% of the infected individuals become chronic HCV carriers with an increased risk of developing HCC. Oncoviruses associated with neoplasms in humans are either DNA viruses or retroviruses that synthesize a DNA copy of their RNA genomes during replication using reverse transcriptase. Such viral genomes either integrate into the host cell genome or coreplicate with the host cell DNA. The RNA genome of HCV does not code for a reverse transcriptase, however, and in the absence of a DNA intermediate, it does not integrate into the host cell DNA, and it does not coreplicate as an extrachromosomal episome in concert with the host cell genome, either. Thus, the HCV genome is not subject of the epigenetic machinery of the host cell, and the virus persists in the absence of a latent form, by continuous productive replication in the liver. It is interesting to note that HCV also infects B cells and increases the mutation frequency of *IgH, BCL-6, TP53*, and *β-catenin* genes in in vitro infected B-cell lines, PBMCs, lymphomas, and HCCs by inducing the error-prone DNA polymerase ζ, polymerase ι, and activation-induced cytidine deaminase (Machida et al. 2004). Machida et al. suggested that HCV induces a mutator phenotype that may contribute to the development of HCV-associated oligoclonal lymphoproliferative disorders and HCCs in a “hit and run” tumorigenesis scenario. Therapeutic failure during the standard administration of pegylated interferon-alpha (IFN-α) to HCV-infected patients was traditionally attributed to viral interference with IFN-α signal transduction, i.e., blocking of the JAK–STAT pathway or disruption of type I IFN receptors (Duong et al. 2006).

Recently, however, it was demonstrated that epigenetic silencing of IFN-stimulated genes may also cause IFN resistance in cells harboring HCV replicons (Naka et al. 2006; see also Chap. 14).

A potential mechanism for HCV-mediated inactivation of cellular genes could be promoter hypermethylation because the HCV core protein is capable to activate DNMT1 and DNMT3B (Arora et al. 2008). Such a mechanism could repress the *E-cadherin* promoter, and, in principle, it could mediate the silencing of a series of tumor suppressor genes in HCV-associated HCC (Yang et al. 2003; Li et al. 2004; Narimatsu et al. 2004; Arora et al. 2008). Ko et al. observed that the *SOCS-1* gene coding for the intracellular protein Socs1 that acts as a negative regulator of the JAK/STAT signaling pathway is frequently hypermethylated in HCV-associated HCCs but not so much in HBV-associated tumors (Ko et al. 2008). Increased methylation frequencies of the tumor suppressor genes *P16INK4A*, *RASSF1A, APC*, and *RIZ1* as well as *GSTP1* (glutathione S-transferase P1) were also reported in HCV-positive, HBV-negative HCCs versus nontumorous tissues (Formeister et al. 2010). In parallel, a global hypomethylation of the tumor cell genome was observed, based on increased levels of hypomethylated LINE-1 repetitive sequences. Based on these data, Formeister et al. argued that aberrant CpG methylation plays a role in the pathobiology of HCV-positive HCCs. Yang et al. also observed that *APC* methylation was more frequent in HCV-positive HCCs than in virus-negative tumors (Yang et al. 2003). They also described frequent methylation of SOCS-1, in accordance with the data of Ko et al. (2008; Yang et al. 2003). In addition, Yang et al. reported a frequent methylation of *p15*, a gene encoding a cyclin-dependent kinase inhibitor in HCV-associated hepatocarcinomas (Yang et al. 2003). Decreased expression and hypermethylation of *Gadd45β*, coding for a member of the growth arrest and DNA damage family of proteins involved in stress responses, were observed in HCV-transgenic mice. This observation suggests that downregulation of Gadd45β expression in HCV-associated liver cancer may occur by a similar mechanism (Higgs et al. 2010). These data demonstrate the concurrent methylation of a set of tumor suppressor genes and other cancer-related genes in HCV-associated HCCs, reflecting the epigenetic reprogramming of the hepatocyte genome during hepatocarcinogenesis.

8.7 Epigenetic Changes in Human Papillomavirus-Associated Tumors

Human papillomaviruses (HPVs) are dsDNA viruses associated with a variety of benign epithelial proliferations. Specific high-risk HPVs (HPV-16, HPV-18, HPV-31, HPV-33, and HPV-45), however, are the causative agents of cervical carcinoma (zur Hausen 2002). Since their genome is relatively small, papillomaviruses use the host DNA synthesizing machinery to replicate their genome. The episomal viral genome frequently integrates into the host cell DNA during the carcinogenetic process. E6 and E7, the main oncoproteins encoded by high risk HPVs, are involved not only in the initiation of cervical carcinogenesis, but their persistent expression appears to be necessary for the successive steps of neoplastic development as well.

E6 proteins encoded by high-risk HPVs target the p53 tumor suppressor protein for degradation, whereas high-risk HPV E7 oncoproteins block the function of the retinoblastoma protein (pRB) involved in the regulation of the cell cycle. In addition, both E6 and E7 associate with a series of other cellular proteins as well and thereby modulate key cellular processes (reviewed by Howie et al. 2009; McLaughlin-Drubin and Munger 2009)

8.7.1 HPV: Basic Facts and Host Cell-Dependent Methylomes of the Viral Genome

Although infections with high-risk HPV strains (especially types 16 and 18) are causally related to the development of cervical cancer, only a fraction of the HPV-positive premalignant lesions appear to progress to invasive cancer (reviewed by Szalmas and Konya 2009). Genetic instability was regularly observed during HPV-induced carcinogenesis. This phenomenon is associated with the E7-initiated abnormal centrosome synthesis resulting in multipolar mitotic spindles and abnormal chromosome segregation already in an early phase of cervical carcinogenesis (Duensing and Munger 2003).

The regulatory sequences of the early genome region of HPV are located to the long control region (LCR). The transcription factor AP-1 binds to the LCR and activates the transcription of the early genes including E6 and E7. The LCR appears to be a subject of epigenetic regulation, because ectopic expression of retinoic acid receptor beta 2 (RARβ2) downregulates HPV-18 transcription by abrogating AP-1 binding and targets the LCR for de novo methylation (De Castro Arce et al. 2007). Fernandez et al. determined the DNA methylation pattern of the whole genome ("the methylome") of the high-risk HPVs HPV-16 and HPV-18 in a collection of human cervical samples corresponding to progressive disease stages, such as specimens from asymptomatic carriers, cervical intraepithelial neoplasias, and primary cervical carcinomas. They observed that the genome of HPV-16 and HPV-18 undergoes progressive de novo methylation during the successive phases of tumorigenesis (Fernandez et al. 2009). The viral genome was unmethylated or hypomethylated in samples from asymptomatic carriers, there was a low level of CpG methylation in cervical intraepithelial neoplasias considered to be premalignant lesions, and a moderate methylation level was observed in primary cervix carcinomas. The highest DNA methylation levels were found in four established cervix carcinoma cell lines cultivated in vitro.

8.7.2 Dysregulation of Cellular Epigenetic Processes by the Viral Oncoproteins E6 and E7

The E7 oncoprotein of HPV-16 may induce hypermethylation of selected cellular promoters both directly, by binding to DNMT1 and stimulating its activity

(Burgers et al. 2007), and indirectly, by releasing the so-called "activating" E2F transcription factors that are complexed with members of the retinoblastoma pocket protein family. The released E2F transcription factors not only control the coordinated transcription of genes involved in DNA replication and cell cycle progression but activate the expression of the *DNMT1* gene as well (Kimura et al. 2003; Iaquinta and Lees 2007). These mechanisms may contribute to local hypermethylation of a set of cellular promoters in HPV-16- and HPV-18-infected cells (reviewed by Szalmas and Konya 2009; Wentzensen et al. 2009).

One of the genes silenced by CpG methylation, *cyclin A1* (*CCNA1*), was considered a candidate tumor marker for the early diagnosis of HPV-associated invasive cervical cancer (Kitkumthorn et al. 2006). In a follow-up study, however, no correlation was found between the quantity of HPV and *CCNA1* promoter hypermethylation. It was observed that CCNA1 methylation was associated with the presence of integrated HPV genomes, rather than the episomal form (Yanatatsaneejit et al. 2011). Hypermethylation of the *APC* promoter was preferentially associated with a distinct histological type, adenocarcinoma (Dong et al. 2001; Wisman et al. 2006; Wentzensen et al. 2009). Methylation of *CDKN2B, RASSF1A, TIMP3*, and *TP73* was also more frequent in cervical adenocarcinomas (Henken et al. 2007). Wentzensen et al. found, based on the data of several studies, that the weighted mean methylation frequency of *TIMP3* as well as *HIC1* was considerably higher in adenocarcinoma than in squamous cell carcinoma (Wentzensen et al. 2009). In contrast, *PAX1* methylation appeared to be specific for squamous cell carcinoma (Lai et al. 2008). In addition, *DAPK1* and *CADM1* were also identified as histotype-specific markers because they were found to be significantly more frequently methylated in squamous cell carcinomas than in adenocarcinomas (Henken et al. 2007). In addition, *DAPK1* and *CADM1*, as well as *RARB*, consistently showed elevated methylations in cervical cancers across studies (Wentzensen et al. 2009). Henken et al. suggested that the *MGMT* promoter could be regarded as a *common marker* of cervical neoplasms because it was frequently methylated both in cervical adenocarcinomas and squamous cell carcinomas (Henken et al. 2007). A comparison of the reported MGMT methylation frequencies, however, showed a wide range variation among the individual studies (Wentzensen et al. 2009). Recently, Lai et al. determined the methylation levels of four genes (*SOX1, PAX1, LMX1A*, and *NKX6-1*) coding for transcription factors using a quantitative methylation polymerase chain reaction. They found a very low level of methylation in normal uterine cervix and cervical intraepithelial neoplasm types 1 and 2 (CIN1 and CIN2) (Lai et al. 2010). CIN1 and CIN2 progress to invasive cancer with a low probability. In contrast, cervical intraepithelial neoplasm 3 (CIN3) and carcinoma in situ (CIS) that progress to invasive cancer with a higher probability displayed significantly higher methylation levels of the four indicator genes, similarly to squamous cell carcinomas and adenocarcinomas of the cervix (Lai et al. 2010). Although the role of the SOX1 (sex-determining region Y, box 1), PAX1 (paired box gene 1), LMX1A (LIM homeobox transcription factor 1 α), and NKX6-1 (NK6 transcription factor-related locus 1) in the physiology of cervical epithelial cells and cervical carcinogenesis remains to be explored, assessing the DNA methylation level of their genes may be applicable in the detection of CIN3 and worse (CIN+) lesions.

The accumulation of frequent methylation events involving five candidate tumor suppressor genes (*TP73, ESR1, RARβ, DAPK1*, and *MGMT*) was also observed in an in vitro model system of cervical carcinogenesis (Henken et al. 2007). De novo methylation of *TP73* and *ESR1* started soon after primary keratinocyte cell lines carrying transfected HPV genomes became immortal. *ESR1* encodes the *estrogen receptor* that was found to be downregulated early in HPV-infected cervical dysplasia (Bekkers et al. 2005). Methylation of *RARβ* and *DAPK1* became manifest in late immortal passages, whereas methylation of *MGMT* followed the acquisition of anchorage independence (Henken et al. 2007). In contrast, no methylation was evident in preimmortal HPV-18-transfected cells characterized by extended but still finite lifespan. It is noteworthy that despite E7 expression in preimmortal cells and the known capacity of E7 to induce de novo methylation via the upregulation and activation of DNMT1, none of the 29 genes analyzed was hypermethylated at the preimmortal stage. Thus, Henken et al. speculated that the accumulation of five methylated, inactivated genes following immortalization is associated with a growth advantage of the HPV-containing keratinocytes, provided by switching off these particular genes.

In addition to regional DNA hypermethylation, a global hypomethylation of the cervical cell genome was also observed in cervix carcinomas. The level of global hypomethylation was higher in invasive cancer than in normal cervical samples or intraepithelial neoplasia (CIN1, CIN2, or CIN3) (Flatley et al. 2009). The normally methylated promoter CGIs of *CAGE* (cancer/testis antigen), a negative regulator of p53 expression, was found to be frequently hypomethylated in cervical squamous cell carcinoma (Lee et al. 2006).

E7 is a pleiotropic regulator involved not only in gene silencing but also in gene activation. In human keratinocytes, HPV-16 E7 increased histone H3 acetylation at the *E2F1* and *CDC25A* promoters (Zhang et al. 2004). In keratinocytes expressing HPV-16 E6/E7 proteins, a global decrease of H3K27me3 levels was observed, in spite of the parallel increase of the PRC2 histone lysine methyltransferase EZH2 that trimethylates lysine 27 of histone H3 (Hyland et al. 2011). This paradox phenomenon was explained by the concurrent upregulation of the KDM6A lysine demethylase, removing the methyl groups from lysine 27 in H3K27me3. In addition, the PRC1 protein Bmi-1 was downregulated. All of these changes resulted in the derepression of HOX genes. Activation of EZH2 expression by HPV-16 E7—via the E7-mediated release of E2F from pocket proteins—was also observed in HPV-positive tumor cells by Holland et al., who demonstrated that EZH2 facilitated cell cycle progression at the G1–S boundary and contributed to the apoptotic resistance of the cells, too (Holland et al. 2008).

Although the contribution of E6 versus E7 to these complex epigenetic changes remains to be elucidated, recent data by Hsu et al. suggested that E6 interacted with the arginine methyltransferases CARM1 and PRMT1 and the lysine methyltransferase SET7, downregulating their enzymatic activities (Hsu et al. 2011). E6 blocked histone methylation catalyzed by CARM1 and PRMT1 at p53-responsive promoters resulting in suppression of p53 downstream genes. McLaughlin-Drubin et al. described that HPV-16 E7 expression caused a significant reduction of the

H3K27me3 repressive mark in primary human epithelial cells. E7 induced the lysine demethylases KDM6A and KDM6B that target histone H3K27me3 and thereby disrupt Polycomb repressor complexes. In addition, KDM6B upregulated p16INK4A, a CDK4/6 inhibitor involved in oncogenic stress-induced senescence. E7 simultaneously blocks, however, pRB, a key mediator of p16INK4A-induced senescence, permitting thereby continuous cell proliferation. Epigenetic reprogramming by E7 also resulted in the activation of the homeobox genes *HOXC5* and *HOXC8* in primary human epithelial cells (McLaughlin-Drubin et al. 2011). Because *HOX* genes are involved in the regulation of epidermal differentiation, their derepression may alter the phenotype and behavior of the cells. McLaughlin-Drubin et al. speculated that due to epigenetic reprogramming, a more stem cell-like state is achieved by E7.

8.8 Tumor-Antigen-Mediated Silencing of a Master Regulator Gene in Merkel Cell Polyomavirus-Associated Carcinoma of the Skin

The association of polyomaviruses, small dsDNA viruses, with malignant tumors of humans remains to be firmly established, with the exception of Merkel cell polyomavirus, in spite of the fact that BK virus, a human polyomavirus, is capable to induce tumors in experimental animals (ter Schegget et al. 1980). The large T antigen (tumor antigen, TAg) of BK virus elicits malignant transformation of cells in vitro by inhibiting the retinoblastoma (Rb) protein family as well as the p53 tumor suppressor protein (Helt and Galloway 2003). Interaction of TAg with Rb disrupted Rb/E2F complexes resulting in the activation of a set of promoters with E2F-binding sites, including the *DNMT1* promoter in human prostate epithelial cells (McCabe et al. 2006). In a transgenic mouse model of prostate carcinogenesis (TRAMP), expression of the related simian polyomavirus SV40 TAg also elevated the expression of the murine Dnmt1 enzyme in the developing prostatic intraepithelial neoplasias (PIN) as well as prostate carcinomas and their metastases. Treatment with the Dnmt1 inhibitor 5-aza-2′-deoxycytidine prevented the progression of PIN lesions to malignant disease (McCabe et al. 2006). These data indicated that induction of DNMT activity by a polyomavirus TAg was an early event required for malignant transformation and tumorigenesis in the TRAMP model. Lytic replication of BK virus may cause nephropathy in renal transplant patients. The viral DNA appears to be unmethylated during productive viral replication (Chang et al. 2011).

Merkel cell polyomavirus (MCPyV) was discovered in Merkel cell carcinoma cells arising from specialized cells mediating mechanotransduction in touch-sensitive areas of the epidermis (Feng et al. 2008). According to lineage-tracing experiments, Merkel cells arise through the differentiation of epidermal progenitors during embryonic development, a process regulated by the basic helix-loop-helix transcription factor atonal homologue 1 (Atoh1, also called Math1 or Hath1, Van Keymeulen

et al. 2009). In *Drosophila*, the corresponding atonal (Ato) protein acts as a master regulator of cell fate specification, regulating the formation and progression of retinal tumors (Bossuyt et al. 2009a). Loss of *Atoh1* promoted tumor formation in mouse models of colorectal cancer, and expression of the human *ATOH1* was reduced, due to deletion or inactivation by CpG methylation, in Merkel cell carcinomas as well as colorectal carcinomas (Bossuyt et al. 2009b). The promoter of the tumor suppressor gene *RASSF1A* was also hypermethylated in about half of the MCC samples (Helmbold et al. 2009). These observations indicate that epigenetic or genetic inactivation of a conserved master regulator gene contributes to the pathogenesis of MCPyV-associated Merkel cell carcinoma.

8.9 Conclusions and Perspectives

Alterations of the host cell epigenome regularly accompany virus-induced tumorigenesis in humans, and the epigenetic changes can be directly related to the viral oncoproteins that act as epigenetic modifiers. In addition to oncoproteins, virus-encoded or virus-induced nontranslated RNA molecules, including long noncoding RNAs (lncRNAs), may also influence, in principle, the gene expression pattern of the host cell, similarly to the cellular lncRNA HOTAIR that may retarget PRC2 to more than 800 new sites, leading to an altered pattern of histone modification and gene expression (Gupta et al. 2010, reviewed by Hung and Chang 2010). In addition to the RNA polymerase III-transcribed EBER1 and EBER2 and the microRNAs processed from EBV transcripts, EBV encodes also v-snoRNA1, a relative of small nucleolar RNAs (snoRNAs) (Hutzinger et al. 2009). The nontranslated v-snoRNA1 was expressed in EBV-positive LCLs and Burkitt's lymphoma cells, but it was absent from EBV-negative cells. Because LMP1 and LMP2A, the EBV proteins upregulating DNMTs, are not expressed in latency type I Burkitt's lymphoma cells, one may speculate that either EBNA1 or a nontranslated EBV RNA like v-snoRNA1 may mediate the change of the cellular epigenotype observed in Burkitt's lymphoma cells (see Sect. 2.3.1). In LCLs carrying latent EBV genomes, cellular noncoding RNAs upregulated by the virus (Mrazek et al. 2007) may also contribute to the epigenetic reprogramming of the host cell. It is worthy to mention that KSHV expresses a noncoding RNA, called PAN (polyadenylated nuclear RNA), during productive infection that may interfere with the immune response (Rossetto and Pari 2011), and expression of the viral noncoding RNA HSUR1 in T cells transformed by the primate gammaherpesvirus *Herpesvirus saimiri* may downregulate a cellular miRNA in a sequence-specific manner (Cazalla et al. 2010).

The data accumulated regarding the epigenotypes of human tumor-associated viruses and their host cells may allow the development of new diagnostic methods for the detection and monitoring of virus-associated neoplasms. Novel epigenetic therapies are either at the stage of preclinical studies or, as in the case of certain

HDAC inhibitors administered to patients with cervical carcinoma, at the stage of clinical trials already (De la Cruz-Hernandez et al. 2011; Takai et al. 2011).

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