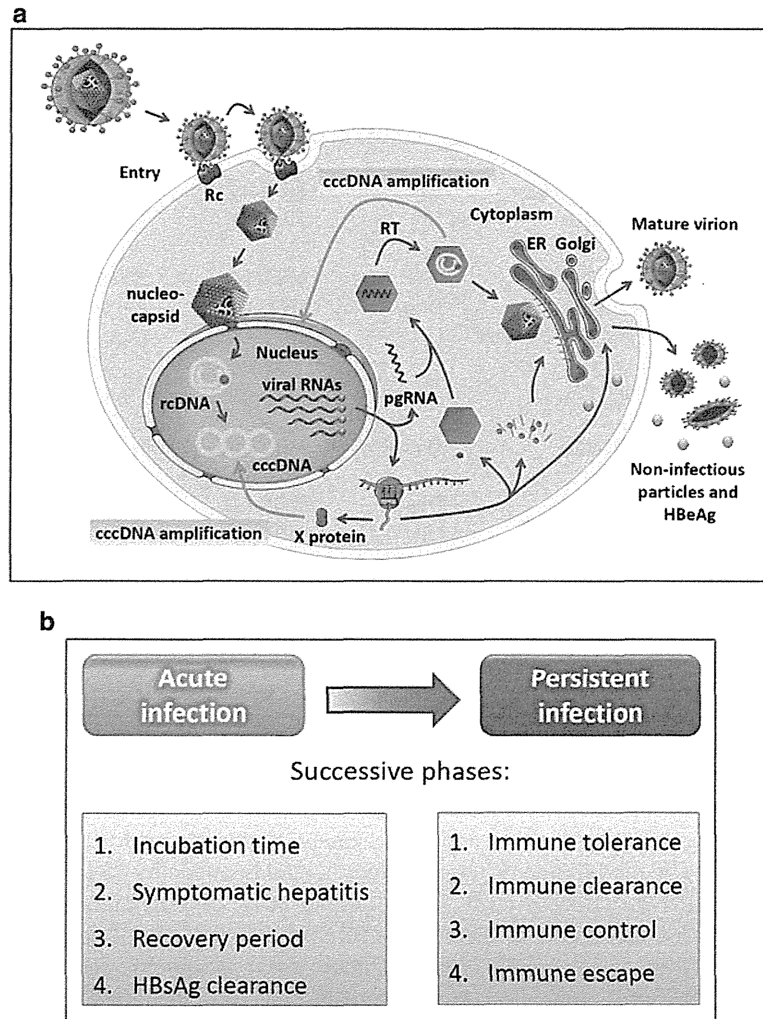

Fig. 7.5 The HBV infection (**a**) Schematic representation of the HBV life cycle. (**b**) HBV natural history of infection. Abbreviations: *cccDNA* covalently closed circular DNA, *ER* endoplasmic reticulum, *HBeAg* hepatitis B extracellular "e" antigen, *HBsAg* HBV surface antigen, *pgRNA* pregenomic RNA, *Rc* receptor, *rcDNA* relaxed circular DNA, *RT* reverse transcription

6.2 MiRNAs Involved in the Regulation of HBV Gene Expression, Replication and Effects on the Carcinogenesis

Viruses, nuclear DNA viruses in particular, need some time to complete their life cycle. During this period, the host cell can develop defense mechanisms such as cell cycle arrest and viral clearance. By taking advantage of the cellular miRNA machinery,

Table 7.2 Cellular miRNAs and their effects on HBV biology, pathogenesis or related-HCC HBV (↑): Promotes HBV replication, HBV (↓): Inhibits HBV replication, HCC (↑): Development and/or growth of HCC

Target genes	miRNAs	miRNA expressions	HBV or HCC status	Reference
Viral target genes				
HBsAg	miR-199-3p	Up	HBV(↓)	Zhang et al. (2010)
HBVpre-S1	miR-210	Up	HBV(↓)	Zhang et al. (2010)
Cellular target genes				
HDAC4	miR-1	Up	HBV (↑)	Zhang et al. (2011a)
c-myb	miR-15a	Down	HCC (↑)	Liu et al. (2009)
E2F1 (c-myc repressor)	miR-17-92 cluster	Up	HCC (↑)	Connolly et al. (2008)
PTEN (?)	miR-21	Up	HCC (↑)	Connolly et al. (2008)
cyclin G1 (p53 modulator)	miR-122	Down	HBV (↑), HCC (↑)	Wang et al. (2012)
DNMT1	miR-152	Down	HBV (↓)	Huang et al. (2010)
SOCS1 (STAT inhibitor)	miR-155	Up	HBV(↓)	Su et al. (2011)
HLA-A (miR-181)	miR-181a, -181b, 200b	Up	HBV (↑)	Liu et al. (2009)
NFIB	miR-372,-373	Up	HBV (↑)	Guo et al. (2011)
STAT3	let-7 family	Down	HBV (↑?), HCC (↑)	Wang et al. (2010)

these viruses can more easily and efficiently help to promote a favorable cellular environment for viral replication and achievement of the life cycle (Skalsky and Cullen 2010). The modulation of the machinery could be made by direct action on the cellular miRNAs (Backes et al. 2012; Jopling et al. 2005) (inhibition or up-regulation) or by expression of their own miRNAs that will mimic their cellular counterparts (Gottwein et al. 2007; Lu and Cullen 2004). Despite the fact that HBV is a nuclear DNA virus, none viral-encoded miRNA has been identified so far. Only one putative HBV-miRNA, with hypothetical regulation role on its own genome, was deduced by computational approach (Jin et al. 2007). However, several cellular miRNAs are involved in the HBV viral replication. They are presented here above and summarized in Table 7.2.

6.2.1 Cellular miRNAs That Promote HBV Replication

MiR-1 can enhance the HBV core promoter transcription and thus increase the viral replication by modulating the expression of several host genes such as transcription factors (Zhang et al. 2011a). The report has confirmed that the histone deacetylase 4 (HDAC4) expression is down-regulated by miR-1. Knowing that the cccDNA amplification is controlled by epigenetic regulation (Pollicino

et al. 2006), miR-1 could act in complementarity with the nuclear HBV X protein (HBx) in order to induce these modifications (Belloni et al. 2009). However, miR-1 can also inhibit the cell proliferation and even induce a reverse cancer cell phenotype (Zhang et al. 2011a). The roles of miR-1 in the cell proliferation and hepatocellular carcinogenesis (Datta et al. 2008) seem to be contradictory with the viral replication and with the characteristics of oncogenic virus but must represent benefit for HBV survival.

Another miRNA, miR-501, has also been suggested to work together with HBx for the benefit of viral replication (Jin et al. 2013). HBx itself has also the ability to deregulate the cellular miRNAs expression. This small protein is a key regulator of HBV infection. It is usually over-expressed in HCC and accumulated evidence indicates that HBx can promote hepatocarcinogenesis by disrupting the normal physiologic mechanisms of the host cell (Chirillo et al. 1997; Lee et al. 2005; Tian et al. 2013). The let-7 family of miRNAs has been demonstrated to be negatively regulated by HBx (Wang et al. 2010). This miRNA family is often observed down-regulated in many cancers including HCC (Guo et al. 2006; Johnson et al. 2005; Yu et al. 2007). The consequence of this down-regulation is the increase activity of that signal transducer and activator of transcription 3 (STAT3) that supports the cell proliferation, and potentially the hepatocarcinogenesis.

Finally, the miRNAs can promote the viral replication by the indirect stimulation the HBV enhancer element I or II. It is the case for the CCAAT/enhancer binding protein that binds and activates the HBV enhancer II in a dose-dependent manner (Lopez-Cabrera et al. 1991). miR-372, together with miR-373, targets the nuclear factor I/B, an important regulator of several viruses (Nagata et al. 1983), and so supports the HBV expression (Guo et al. 2011).

6.2.2 Cellular miRNAs That Prevent HBV Replication

One of the best studied miRNAs in liver-related diseases is miR-122. This liver-specific miRNA is expressed at high levels in normal hepatocytes (about 70 % of the total miRNA population in the adult liver) (Lagos-Quintana et al. 2002) and is pivotal in numerous aspects of the liver function such as lipid metabolism, liver development, differentiation, growth and neoplastic transformation (Girard et al. 2008). The essential role of mir-122 in the HCV replication reflects furthermore the importance of this miRNA in the infection process (Jopling et al. 2005). While the loss of miR-122 expression is impeding HCV replication, it is enhancing the replication in the circumstance of HBV infection (Wang et al. 2012). In fact, miR-122 can negatively regulate the viral gene expression and replication by direct binding to a highly conserved sequence of HBV (Chen et al. 2011). This repression effect can apparently be impeded by a negative feedback loop involving the Heme oxygenase-1 (Qiu et al. 2010). A recent study has reported the indirect implication of HBx in miR-122 deregulation (Song et al. 2013) that could, at least partially, explain the difference observed between the two viruses. Knowing that miR-122 expression is low in HBV and HCC tissues (Wang et al. 2012; Kutay et al. 2006)

and that HBV replication is usually low or absent in HCC cells (Wong et al. 2006), miR-122 is a highly potential linker between HBV infection and liver carcinogenesis (Wang et al. 2012; Fan et al. 2011) and therefore a predilected target for future clinical applications.

The miR-17-92 cluster is also important in the HBV-associated HCC. This polycistron includes six miRNAs (miR-17-5p, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a-1) and its up-regulated expression is associated with malignancies (Hayashita et al. 2005). By using human HBV-positive human HCC tissues, hepatoma cell lines and woodchuck hepatitis virus -induced HCC animal model (Popper et al. 1987), Connolly and colleagues were able to demonstrate the elevated expression of miR-17-92 cluster and its implication in the malignant phenotype (Connolly et al. 2008). The expression could be amplified by c-myc activation (He et al. 2005), under HBx control (Terradillos et al. 1997), to contribute to HBV latency state (Jung et al. 2013). The consequence is the induction of liver oncogenesis. Since the RNA intermediates of HBV (pgRNA and transcripts) are good targets of miRNA action, it is not surprising to observe several cellular miRNAs with different binding sites. So, in addition to miR-122 that targets the polymerase region (Chen et al. 2011), the mir-199a-3p and mir-210 can repress the S and pre-S1 regions, respectively (Zhang et al. 2010).

All the examples illustrating cellular miRNAs as inhibitors of the viral replication are a bit difficult to comprehend initially because of their obvious negative effect on HBV infection. However, it can be understood by keeping in mind the survival of the virus into the host organism. The natural history of HBV infection shows often a transition from acute to chronic infection, especially in young children. This step corresponds to a failure of the immune system to eradicate the virus (Fig. 7.5b). One of the escape pathways is the successful adaptation to the immune-induced down-regulation of replication. The virus could evade the immune system by reaching a dormant state into the infected hepatocytes, under the cccDNA form, and survive until its eventual life cycle reactivation (Ganem and Prince 2004; Belloni et al. 2009, 2012; Huang et al. 2010). The study of Huang and colleagues reports the CpG islands methylation of the cccDNA by the DNA methyltransferase 1 (DNMT1) to prevent the viral gene expression and therefore the viral antigen presentation. DNMT1 over-expression is induced by a decrease of miR-152, under the effect of HBx (Huang et al. 2010).

6.3 MiRNAs in the Modulation of the Immune System and Effects on the Carcinogenesis

HBV must adapt to a very complex network in order to survive. It has to cope with the modification of homeostasis, the cell cycle arrest, the apoptosis and the destruction of the host cell by the immune cells. MiRNAs are also important in the development and function of immune system (Baltimore et al. 2008). Some miRNAs in particular are crucial for modulating innate and adaptive immune responses. MiR-155 has multi-roles during an innate immune response such as the regulation of the acute inflammatory response after recognition of pathogens by the toll-like

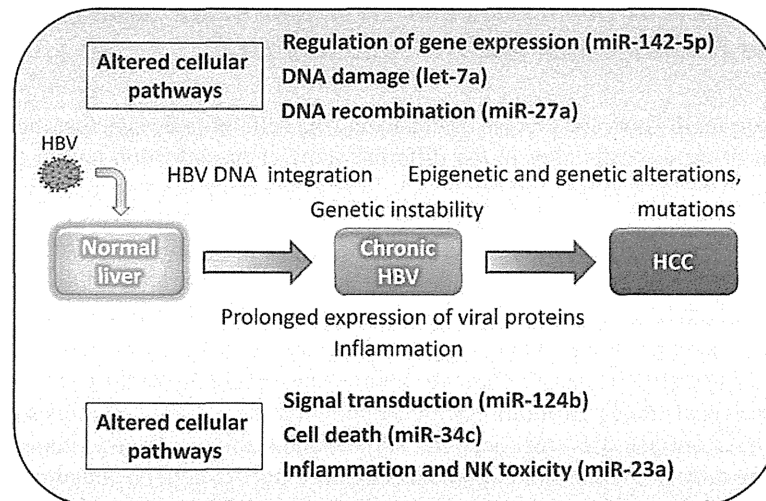

Fig. 7.6 Chronology of events from the HBV infection until HCC development. The indicated altered miRNAs and related pathways are based on the results from Ura et al. (2009)

receptors (O'Connell et al. 2007; Tili et al. 2007). The up-regulation of miR-155 can lead to prolonged exposure to inflammation, a well-known causal agent to cancers like HCC (Berasain et al. 2009). Two recent studies suggest a role of miR-155 in hepatocarcinogenesis and HBV infection (Table 7.2). Using HCC-induced mouse model, Wang and collaborators have demonstrated an oncogenic role of miR-155 at the early stages of the tumorigenesis (Wang et al. 2009a). On the other hand, the ectopic expression of miR-155 in human hepatoma cells enhances the innate immunity through promotion of the JAK/STAT pathway and down-regulates HBx expression (Su et al. 2011).

A study analyzing the modified expression profiles of miRNAs in a stable HBV-expressing cell line revealed the up-regulation of miR-181a (Liu et al. 2009) (Table 7.2). The deregulation of this miRNA in liver cell might participate to the establishment of HBV persistence through inhibition of the human leukocyte antigen A (HLA-A) -dependent HBV antigen presentation. To date, it is unclear if miRNAs altered in the host cell, like miR-181a and miR-146a also present in Liu's study, miRNAs involved in ubiquitous and cell-specific regulatory functions, could affect directly the immune cells. The presence of circulating miRNAs, as well as the existence of intercellular nanovesicle-mediated miRNA transfer and its impact on the environmental modulation, could potentially support that hypothesis (Arataki et al. 2013; Waidmann et al. 2012; Li et al. 2010, 2012; Zhou et al. 2011; Kogure et al. 2011). The current knowledge shows an altered miRNA profile expression between normal and HCC liver at the different stages, and between the HBV and HCV-induced HCC (Murakami et al. 2006; Li et al. 2008; Budhu et al. 2008; Ura et al. 2009). For the latest one, this reflects the variation in the cellular pathways that are modulated as a consequence of the viral infection (Fig. 7.6).

6.4 MiRNAs as Biomarkers and Treatment-Based Strategies for HBV Infection and HBV-Induced HCC

It is important to know the precise mechanisms, the cellular pathways that the viral infection or cancer cells alter in the different steps of the infection and/or tumor evolution. The knowledge will allow developing powerful targeted therapeutical strategies. The significance of miRNAs in antiviral immunity and liver carcinogenesis emphasizes their values as therapeutic targets for HBV infection and HBV-induced HCC. MiR-122 and miR-18a are of particular interest. They are both released in the blood and could be used as potential non-invasive biomarkers for HBV-related HCC screening (Liu et al. 2009; Waidmann et al. 2012; Li et al. 2012). Some other reports suggest using a miRNA panel in order to improve the specificity of the test (Li et al. 2010; Zhou et al. 2011). In addition with the current routinely used markers such as HBV surface antigen, HBV extracellular antigen and alanine aminotransferase, the circulating miRNAs represent a significant clinical value for better evaluation of the HBV-infection status, liver injury and early diagnosis of HCC.

In the therapeutic perspective, the work of Ura's group is valuable. They analyzed the livers of HBV and HCV positive patients with HCC to identify the miRNAs that are differentially expressed. Nineteen miRNAs were clearly differentiated between HBV and HCV groups, six specific for HBV and thirteen specific for HCV. Based on the miRNAs profile, they made a pathway analysis of candidate targeted genes and were also able to distinguish the cellular mechanisms altered in HBV or HCV-infected livers (Ura et al. 2009). The HBV infection alters mostly the pathways related to signal transduction, inflammation and natural killer toxicity, DNA damage, recombination, and cell death (Fig. 7.6), while HCV infection modifies those involved in immune response involving antigen presentation, cell cycle and cell adhesion (Ura et al. 2009).

Moreover, technological advances in the delivery of miRNA and RNA interference enable safe and efficient *in vivo* miRNA gene therapy, as exemplify by the recent study from Kota and collaborators on the liver cancer (Kota et al. 2009). They used an adeno-associated virus to deliver miR-26a in a mouse model of HCC. This resulted in the successful inhibition of the cancer cell proliferation, induction of the tumor-specific apoptosis, and protection from disease progression without toxicity.

7 Concluding Remarks

MiRNAs have emerged as novel key players in the control of gene expression in cells. Investigations of their profiling have unveiled specific miRNA deregulations in tumors and in condition of viral infection. On the viral point of view, the deregulated pathways mirror the strategies of the virus to allow its replication and evade the host defense mechanisms to survive. On the cellular point of view, they mirror the immune response that is trying to get rid of the intruder and that become

deregulated. In both cases, the viral infection leads to the alteration of miRNA expression by RSSs that can trigger tumorigenesis. Several oncogenic viruses, especially herpesviruses like EBV and KSHV, encode their own miRNAs to modify both cellular and viral gene expression (Pfeffer et al. 2004). This step is crucial for their latency phase. On the other hand, HPV, HBV and HCV do not express viral miRNAs but can affect the host miRNA pathway. The present and future knowledge about miRNA will broaden our understanding of the pathogenesis of oncogenic viruses and most certainly allow developing efficient oncogenic viral therapies.

Acknowledgements The authors would like to thank Servier Medical Art for their image bank used to create the illustrations.

References

Abend JR, Uldrick T, Ziegelbauer JM (2010) Regulation of tumor necrosis factor-like weak inducer of apoptosis receptor protein (TWEAKR) expression by Kaposi's sarcoma-associated herpesvirus microRNA prevents TWEAK-induced apoptosis and inflammatory cytokine expression. J Virol 84(23):12139–12151

Alajez NM, Lenarduzzi M, Ito E, Hui AB, Shi W, Bruce J et al (2011) MiR-218 suppresses nasopharyngeal cancer progression through downregulation of survivin and the SLIT2-ROBO1 pathway. Cancer Res 71(6):2381–2391

Amoroso R, Fitzsimmons L, Thomas WA, Kelly GL, Rowe M, Bell AI (2011) Quantitative studies of Epstein-Barr virus-encoded microRNAs provide novel insights into their regulation. J Virol 85(2):996–1010

Appel N, Schaller T, Penin F, Bartenschlager R (2006) From structure to function: new insights into hepatitis C virus RNA replication. J Biol Chem 281(15):9833–9836

Arataki K, Hayes CN, Akamatsu S, Akiyama R, Abe H, Tsuge M et al (2013) Circulating microRNA-22 correlates with microRNA-122 and represents viral replication and liver injury in patients with chronic hepatitis B. J Med Virol 85(5):789–798

Au Yeung CL, Tsang TY, Yau PL, Kwok TT (2011) Human papillomavirus type 16 E6 induces cervical cancer cell migration through the p53/microRNA-23b/urokinase-type plasminogen activator pathway. Oncogene 30(21):2401–2410

Backes S, Shapiro JS, Sabin LR, Pham AM, Reyes I, Moss B et al (2012) Degradation of host microRNAs by poxvirus poly(A) polymerase reveals terminal RNA methylation as a protective antiviral mechanism. Cell Host Microbe 12(2):200–210

Baer R, Bankier AT, Biggin MD, Deininger PL, Farrell PJ, Gibson TJ et al (1984) DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature 310(5974):207–211

Bai S, Nasser MW, Wang B, Hsu SH, Datta J, Kutay H et al (2009) MicroRNA-122 inhibits tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib. J Biol Chem 284(46):32015–32027

Baltimore D, Boldin MP, O'Connell RM, Rao DS, Taganov KD (2008) MicroRNAs: new regulators of immune cell development and function. Nat Immunol 9(8):839–845

Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. Cell 136(2):215–233

Barth S, Pfuhl T, Mamiani A, Ehses C, Roemer K, Kremmer E et al (2008) Epstein-Barr virus-encoded microRNA miR-BART2 down-regulates the viral DNA polymerase BALF5. Nucleic Acids Res 36(2):666–675

Bellare P, Ganem D (2009) Regulation of KSHV lytic switch protein expression by a virus-encoded microRNA: an evolutionary adaptation that fine-tunes lytic reactivation. Cell Host Microbe 6(6):570–575

Belloni L, Pollicino T, De Nicola F, Guerrieri F, Raffa G, Fanciulli M et al (2009) Nuclear HBx binds the HBV minichromosome and modifies the epigenetic regulation of cccDNA function. Proc Natl Acad Sci U S A 106(47):19975–19979

Belloni L, Allweiss L, Guerrieri F, Pediconi N, Volz T, Pollicino T et al (2012) IFN-alpha inhibits HBV transcription and replication in cell culture and in humanized mice by targeting the epigenetic regulation of the nuclear cccDNA minichromosome. J Clin Invest 122(2):529–537

Berasain C, Castillo J, Perugorria MJ, Latasa MU, Prieto J, Avila MA (2009) Inflammation and liver cancer: new molecular links. Ann N Y Acad Sci 1155:206–221

Bhanja Chowdhury J, Shrivastava S, Steele R, Di Bisceglie AM, Ray R, Ray RB (2012) Hepatitis C virus infection modulates expression of interferon stimulatory gene IFITM1 by upregulating miR-130A. J Virol 86(18):10221–10225

Bihrer V, Friedrich-Rust M, Kronenberger B, Forestier N, Haupenthal J, Shi Y et al (2011) Serum miR-122 as a biomarker of necroinflammation in patients with chronic hepatitis C virus infection. Am J Gastroenterol 106(9):1663–1669

Boshoff C, Weiss R (2002) AIDS-related malignancies. Nat Rev Cancer 2(5):373–382

Brechot C, Pourcel C, Louise A, Rain B, Tiollais P (1980) Presence of integrated hepatitis B virus DNA sequences in cellular DNA of human hepatocellular carcinoma. Nature 286(5772): 533–535

Budhu A, Jia HL, Forgues M, Liu CG, Goldstein D, Lam A et al (2008) Identification of metastasis-related microRNAs in hepatocellular carcinoma. Hepatology 47(3):897–907

Cai X, Lu S, Zhang Z, Gonzalez CM, Damania B, Cullen BR (2005) Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. Proc Natl Acad Sci U S A 102(15):5570–5575

Cai X, Schafer A, Lu S, Bilello JP, Desrosiers RC, Edwards R (2006) Epstein-Barr virus microRNAs are evolutionarily conserved and differentially expressed. PLoS Pathog 2(3):e23

Cermelli S, Ruggieri A, Marrero JA, Ioannou GN, Beretta L (2011) Circulating microRNAs in patients with chronic hepatitis C and non-alcoholic fatty liver disease. PLoS One 6(8):e23937

Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT et al (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 33(20):e179

Chen SJ, Chen GH, Chen YH, Liu CY, Chang KP, Chang YS et al (2010) Characterization of Epstein-Barr virus miRNAome in nasopharyngeal carcinoma by deep sequencing. PLoS One 5(9) pii: e12745

Chen Y, Shen A, Rider PJ, Yu Y, Wu K, Mu Y et al (2011) A liver-specific microRNA binds to a highly conserved RNA sequence of hepatitis B virus and negatively regulates viral gene expression and replication. FASEB J 25(12):4511–4521

Cheung O, Puri P, Eicken C, Contos MJ, Mirshahi F, Maher JW et al (2008) Nonalcoholic steatohepatitis is associated with altered hepatic microRNA expression. Hepatology 48(6):1810–1820

Chirillo P, Pagano S, Natoli G, Puri PL, Burgio VL, Balsano C et al (1997) The hepatitis B virus X gene induces p53-mediated programmed cell death. Proc Natl Acad Sci U S A 94(15): 8162–8167

Cho WC, Chow AS, Au JS (2009) Restoration of tumour suppressor hsa-miR-145 inhibits cancer cell growth in lung adenocarcinoma patients with epidermal growth factor receptor mutation. Eur J Cancer 45(12):2197–2206

Choy EY, Siu KL, Kok KH, Lung RW, Tsang CM, To KF et al (2008) An Epstein-Barr virus-encoded microRNA targets PUMA to promote host cell survival. J Exp Med 205(11):2551–2560

Christoffersen NR, Shalgi R, Frankel LB, Leucci E, Lees M, Klausen M et al (2010) p53-independent upregulation of miR-34a during oncogene-induced senescence represses MYC. Cell Death Differ 17(2):236–245

Connolly E, Melegari M, Landgraf P, Tchaikovskaya T, Tennant BC, Slagle BL et al (2008) Elevated expression of the miR-17-92 polycistron and miR-21 in hepadnavirus-associated hepatocellular carcinoma contributes to the malignant phenotype. Am J Pathol 173(3):856–864

Cosmopoulos K, Pegtel M, Hawkins J, Moffett H, Novina C, Middeldorp J et al (2009) Comprehensive profiling of Epstein-Barr virus microRNAs in nasopharyngeal carcinoma. J Virol 83(5):2357–2367

Coulouarn C, Factor VM, Andersen JB, Durkin ME, Thorgeirsson SS (2009) Loss of miR-122 expression in liver cancer correlates with suppression of the hepatic phenotype and gain of metastatic properties. Oncogene 28(40):3526–3536

Datta J, Kutay H, Nasser MW, Nuovo GJ, Wang B, Majumder S et al (2008) Methylation mediated silencing of MicroRNA-1 gene and its role in hepatocellular carcinogenesis. Cancer Res 68(13):5049–5058

de Vries W, Berkhout B (2008) RNAi suppressors encoded by pathogenic human viruses. Int J Biochem Cell Biol 40(10):2007–2012

Dolken L, Malterer G, Erhard F, Kothe S, Friedel CC, Suffert G et al (2010) Systematic analysis of viral and cellular microRNA targets in cells latently infected with human gamma-herpesviruses by RISC immunoprecipitation assay. Cell Host Microbe 7(4):324–334

Dreher A, Rossing M, Kaczkowski B, Andersen DK, Larsen TJ, Christophersen MK et al (2011) Differential expression of cellular microRNAs in HPV 11, -16, and -45 transfected cells. Biochem Biophys Res Commun 412(1):20–25

Dyson N, Howley PM, Munger K, Harlow E (1989) The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science 243(4893):934–937

Edwards RH, Marquitz AR, Raab-Traub N (2008) Epstein-Barr virus BART microRNAs are produced from a large intron prior to splicing. J Virol 82(18):9094–9106

Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Pear M et al (2006) miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. Cell Metab 3(2):87–98

Fan CG, Wang CM, Tian C, Wang Y, Li L, Sun WS et al (2011) miR-122 inhibits viral replication and cell proliferation in hepatitis B virus-related hepatocellular carcinoma and targets NDRG3. Oncol Rep 26(5):1281–1286

Feederle R, Haar J, Bernhardt K, Linnstaedt SD, Bannert H, Lips H et al (2011) The members of an Epstein-Barr virus microRNA cluster cooperate to transform B lymphocytes. J Virol 85(19):9801–9810

Fujii T, Taguchi H, Katano H, Mori S, Nakamura T, Nojiri N et al (1999) Seroprevalence of human herpesvirus 8 in human immunodeficiency virus 1-positive and human immunodeficiency virus 1-negative populations in Japan. J Med Virol 57(2):159–162

Gabriely G, Wurdinger T, Kesari S, Esau CC, Burchard J, Linsley PS et al (2008) MicroRNA 21 promotes glioma invasion by targeting matrix metalloproteinase regulators. Mol Cell Biol 28(17):5369–5380

Ganem D, Prince AM (2004) Hepatitis B virus infection–natural history and clinical consequences. N Engl J Med 350(11):1118–1129

Gao P, Tchernyshyov I, Chang TC, Lee YS, Kita K, Ochi T et al (2009) c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. Nature 458(7239):762–765

Gatfield D, Le Martelot G, Vejnar CE, Gerlach D, Schaad O, Fleury-Olela F et al (2009) Integration of microRNA miR-122 in hepatic circadian gene expression. Genes Dev 23(11):1313–1326. 9

Girard M, Jacquemin E, Munnich A, Lyonnet S, Henrion-Caude A (2008) miR-122, a paradigm for the role of microRNAs in the liver. J Hepatol 48(4):648–656

Godshalk SE, Bhaduri-McIntosh S, Slack FJ (2008) Epstein-Barr virus-mediated dysregulation of human microRNA expression. Cell Cycle 7(22):3595–3600

Gonzalez SL, Stremlau M, He X, Basile JR, Munger K (2001) Degradation of the retinoblastoma tumor suppressor by the human papillomavirus type 16 E7 oncoprotein is important for functional inactivation and is separable from proteasomal degradation of E7. J Virol 75(16):7583–7591

Gottwein E, Cullen BR (2010) A human herpesvirus microRNA inhibits p21 expression and attenuates p21-mediated cell cycle arrest. J Virol 84(10):5229–5237

Gottwein E, Mukherjee N, Sachse C, Frenzel C, Majoros WH, Chi JT et al (2007) A viral microRNA functions as an orthologue of cellular miR-155. Nature 450(7172):1096–1099

Gottwein E, Corcoran DL, Mukherjee N, Skalsky RL, Hafner M, Nusbaum JD et al (2011) Viral microRNA targetome of KSHV-infected primary effusion lymphoma cell lines. Cell Host Microbe 10(5):515–526

Gourzones C, Ferrand FR, Amiel C, Verillaud B, Barat A, Guerin M et al (2013) Consistent high concentration of the viral microRNA BART17 in plasma samples from nasopharyngeal carcinoma patients – evidence of non-exosomal transport. Virol J 10:119

Greco D, Kivi N, Qian K, Leivonen SK, Auvinen P, Auvinen E (2011) Human papillomavirus 16 E5 modulates the expression of host microRNAs. PLoS One 6(7):e21646

Grundhoff A, Sullivan CS, Ganem D (2006) A combined computational and microarray-based approach identifies novel microRNAs encoded by human gamma-herpesviruses. RNA 12(5):733–750

Gunasekharan V, Laimins LA (2013) Human papillomaviruses modulate microRNA 145 expression to directly control genome amplification. J Virol 87(10):6037–6043

Guo Y, Chen Y, Ito H, Watanabe A, Ge X, Kodama T et al (2006) Identification and characterization of lin-28 homolog B (LIN28B) in human hepatocellular carcinoma. Gene 384:51–61

Guo H, Liu H, Mitchelson K, Rao H, Luo M, Xie L et al (2011) MicroRNAs-372/373 promote the expression of hepatitis B virus through the targeting of nuclear factor I/B. Hepatology 54(3):808–819

Haecker I, Gay LA, Yang Y, Hu J, Morse AM, McIntyre LM et al (2012) Ago HITS-CLIP expands understanding of Kaposi's sarcoma-associated herpesvirus miRNA function in primary effusion lymphomas. PLoS Pathog 8(8):e1002884

Hayashita Y, Osada H, Tatematsu Y, Yamada H, Yanagisawa K, Tomida S et al (2005) A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. Cancer Res 65(21):9628–9632

He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S et al (2005) A microRNA polycistron as a potential human oncogene. Nature 435(7043):828–833

Henke JI, Goergen D, Zheng J, Song Y, Schuttler CG, Fehr C et al (2008) microRNA-122 stimulates translation of hepatitis C virus RNA. EMBO J 27(24):3300–3310

Hitt MM, Allday MJ, Hara T, Karran L, Jones MD, Busson P et al (1989) EBV gene expression in an NPC-related tumour. EMBO J 8(9):2639–2651

Hou W, Tian Q, Zheng J, Bonkovsky HL (2010) MicroRNA-196 represses Bach1 protein and hepatitis C virus gene expression in human hepatoma cells expressing hepatitis C viral proteins. Hepatology 51(5):1494–1504

Hsu PW, Lin LZ, Hsu SD, Hsu JB, Huang HD (2007) ViTa: prediction of host microRNAs targets on viruses. Nucleic Acids Res 35(Database issue):D381–D385

Hsu SH, Wang B, Kota J, Yu J, Costinean S, Kutay H et al (2012) Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver. J Clin Invest 122(8):2871–2883

Huang J, Wang Y, Guo Y, Sun S (2010) Down-regulated microRNA-152 induces aberrant DNA methylation in hepatitis B virus-related hepatocellular carcinoma by targeting DNA methyltransferase 1. Hepatology 52(1):60–70

Iizasa H, Wulff BE, Alla NR, Maragkakis M, Megraw M, Hatzigeorgiou A et al (2010) Editing of Epstein-Barr virus-encoded BART6 microRNAs controls their dicer targeting and consequently affects viral latency. J Biol Chem 285(43):33358–33370

Ishimoto T, Nagano O, Yae T, Tamada M, Motohara T, Oshima H et al (2011) CD44 variant regulates redox status in cancer cells by stabilizing the xCT subunit of system xc(−) and thereby promotes tumor growth. Cancer Cell 19(3):387–400

Janssen HL, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, Patel K et al (2013) Treatment of HCV infection by targeting microRNA. N Engl J Med 368(18):1685–1694

Jin WB, Wu FL, Kong D, Guo AG (2007) HBV-encoded microRNA candidate and its target. Comput Biol Chem 31(2):124–126

Jin J, Tang S, Xia L, Du R, Xie H, Song J et al (2013) MicroRNA-501 promotes HBV replication by targeting HBXIP. Biochem Biophys Res Commun 430(4):1228–1233

Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A et al (2005) RAS is regulated by the let-7 microRNA family. Cell 120(5):635–647

Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P (2005) Modulation of hepatitis C virus RNA abundance by a liver-specific microRNA. Science 309(5740):1577–1581

Jung YJ, Kim JW, Park SJ, Min BY, Jang ES, Kim NY et al (2013) c-Myc-mediated overexpression of miR-17-92 suppresses replication of hepatitis B virus in human hepatoma cells. J Med Virol 85(6):969–978

Keyes WM, Pecoraro M, Aranda V, Vernersson-Lindahl E, Li W, Vogel H et al (2011) DeltaNp63alpha is an oncogene that targets chromatin remodeler Lsh to drive skin stem cell proliferation and tumorigenesis. Cell Stem Cell 8(2):164–176

Kieff EaR AB (2007) Epstein-Barr virus and its replication. In: Fields virology, 5th edn. Lippincott Williams & Wilkins, Philadelphia

Kim do N, Chae HS, Oh ST, Kang JH, Park CH, Park WS et al (2007) Expression of viral microRNAs in Epstein-Barr virus-associated gastric carcinoma. J Virol 81(2):1033–1036

Kim YJ, Cho SY, Yun CH, Moon YS, Lee TR, Kim SH (2008) Transcriptional activation of Cidec by PPARgamma2 in adipocyte. Biochem Biophys Res Commun 377(1):297–302

Kogure T, Lin WL, Yan IK, Braconi C, Patel T (2011) Intercellular nanovesicle-mediated microRNA transfer: a mechanism of environmental modulation of hepatocellular cancer cell growth. Hepatology 54(4):1237–1248

Kosaka N, Iguchi H, Ochiya T (2010) Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. Cancer Sci 101(10):2087–2092

Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW et al (2009) Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell 137(6):1005–1017

Kutay H, Bai S, Datta J, Motiwala T, Pogribny I, Frankel W et al (2006) Downregulation of miR-122 in the rodent and human hepatocellular carcinomas. J Cell Biochem 99(3):671–678

Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T (2002) Identification of tissue-specific microRNAs from mouse. Curr Biol 12(9):735–739

Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A et al (2007) A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 129(7):1401–1414

Lanford RE, Hildebrandt-Eriksen ES, Petri A, Persson R, Lindow M, Munk ME et al (2010) Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science 327(5962):198–201

Lee AT, Ren J, Wong ET, Ban KH, Lee LA, Lee CG (2005) The hepatitis B virus X protein sensitizes HepG2 cells to UV light-induced DNA damage. J Biol Chem 280(39):33525–33535

Lei X, Bai Z, Ye F, Xie J, Kim CG, Huang Y et al (2010) Regulation of NF-kappaB inhibitor IkappaBalpha and viral replication by a KSHV microRNA. Nat Cell Biol 12(2):193–199

Li W, Xie L, He X, Li J, Tu K, Wei L et al (2008) Diagnostic and prognostic implications of microRNAs in human hepatocellular carcinoma. Int J Cancer 123(7):1616–1622

Li LM, Hu ZB, Zhou ZX, Chen X, Liu FY, Zhang JF et al (2010) Serum microRNA profiles serve as novel biomarkers for HBV infection and diagnosis of HBV-positive hepatocarcinoma. Cancer Res 70(23):9798–9807

Li L, Guo Z, Wang J, Mao Y, Gao Q (2012) Serum miR-18a: a potential marker for hepatitis B virus-related hepatocellular carcinoma screening. Dig Dis Sci 57(11):2910–2916

Lin YT, Kincaid RP, Arasappan D, Dowd SE, Hunicke-Smith SP, Sullivan CS (2010) Small RNA profiling reveals antisense transcription throughout the KSHV genome and novel small RNAs. RNA 16(8):1540–1558

Lin X, Liang D, He Z, Deng Q, Robertson ES, Lan K (2011) miR-K12-7-5p encoded by Kaposi's sarcoma-associated herpesvirus stabilizes the latent state by targeting viral ORF50/RTA. PLoS One 6(1):e16224

Liu Y, Zhao JJ, Wang CM, Li MY, Han P, Wang L et al (2009) Altered expression profiles of microRNAs in a stable hepatitis B virus-expressing cell line. Chin Med J 122(1):10–14

Lo AK, To KF, Lo KW, Lung RW, Hui JW, Liao G et al (2007) Modulation of LMP1 protein expression by EBV-encoded microRNAs. Proc Natl Acad Sci U S A 104(41):16164–16169

Lo AK, Dawson CW, Jin DY, Lo KW (2012) The pathological roles of BART miRNAs in nasopharyngeal carcinoma. J Pathol 227(4):392–403

Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R (1999) Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285(5424):110–113

Lopez-Cabrera M, Letovsky J, Hu KQ, Siddiqui A (1991) Transcriptional factor C/EBP binds to and transactivates the enhancer element II of the hepatitis B virus. Virology 183(2):825–829

Lu S, Cullen BR (2004) Adenovirus VA1 noncoding RNA can inhibit small interfering RNA and microRNA biogenesis. J Virol 78(23):12868–12876

Lu F, Stedman W, Yousef M, Renne R, Lieberman PM (2010) Epigenetic regulation of Kaposi's sarcoma-associated herpesvirus latency by virus-encoded microRNAs that target Rta and the cellular Rbl2-DNMT pathway. J Virol 84(6):2697–2706

Lung RW, Tong JH, Sung YM, Leung PS, Ng DC, Chau SL et al (2009) Modulation of LMP2A expression by a newly identified Epstein-Barr virus-encoded microRNA miR-BART22. Neoplasia 11(11):1174–1184

Machlin ES, Sarnow P, Sagan SM (2011) Masking the 5′ terminal nucleotides of the hepatitis C virus genome by an unconventional microRNA-target RNA complex. Proc Natl Acad Sci U S A 108(8):3193–3198

Marquitz AR, Raab-Traub N (2012) The role of miRNAs and EBV BARTs in NPC. Semin Cancer Biol 22(2):166–172

Marquitz AR, Mathur A, Nam CS, Raab-Traub N (2011) The Epstein-Barr virus BART microRNAs target the pro-apoptotic protein Bim. Virology 412(2):392–400

Martinez I, Gardiner AS, Board KF, Monzon FA, Edwards RP, Khan SA (2008) Human papillomavirus type 16 reduces the expression of microRNA-218 in cervical carcinoma cells. Oncogene 27(18):2575–2582

Melar-New M, Laimins LA (2010) Human papillomaviruses modulate expression of microRNA 203 upon epithelial differentiation to control levels of p63 proteins. J Virol 84(10):5212–5221

Moradpour D, Penin F, Rice CM (2007) Replication of hepatitis C virus. Nat Rev Microbiol 5(6):453–463

Mrazek J, Kreutmayer SB, Grasser FA, Polacek N, Huttenhofer A (2007) Subtractive hybridization identifies novel differentially expressed ncRNA species in EBV-infected human B cells. Nucleic Acids Res 35(10):e73

Murakami Y, Yasuda T, Saigo K, Urashima T, Toyoda H, Okanoue T et al (2006) Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. Oncogene 25(17):2537–2545

Murakami Y, Aly HH, Tajima A, Inoue I, Shimotohno K (2009) Regulation of the hepatitis C virus genome replication by miR-199a. J Hepatol 50(3):453–460

Murakami Y, Tanaka M, Toyoda H, Hayashi K, Kuroda M, Tajima A et al (2010) Hepatic microRNA expression is associated with the response to interferon treatment of chronic hepatitis C. BMC Med Genomics 3:48

Murakami Y, Toyoda H, Tanahashi T, Tanaka J, Kumada T, Yoshioka Y et al (2012) Comprehensive miRNA expression analysis in peripheral blood can diagnose liver disease. PLoS One 7(10):e48366

Nachmani D, Stern-Ginossar N, Sarid R, Mandelboim O (2009) Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells. Cell Host Microbe 5(4):376–385

Nagata K, Guggenheimer RA, Hurwitz J (1983) Specific binding of a cellular DNA replication protein to the origin of replication of adenovirus DNA. Proc Natl Acad Sci U S A 80(20):6177–6181

O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D (2007) MicroRNA-155 is induced during the macrophage inflammatory response. Proc Natl Acad Sci U S A 104(5):1604–1609

Parkin DM (2006) The global health burden of infection-associated cancers in the year 2002. Int J Cancer 118(12):3030–3044

Paterlini-Brechot P, Saigo K, Murakami Y, Chami M, Gozuacik D, Mugnier C et al (2003) Hepatitis B virus-related insertional mutagenesis occurs frequently in human liver cancers and recurrently targets human telomerase gene. Oncogene 22(25):3911–3916

Pegtel DM, Cosmopoulos K, Thorley-Lawson DA, van Eijndhoven MA, Hopmans ES, Lindenberg JL et al (2010) Functional delivery of viral miRNAs via exosomes. Proc Natl Acad Sci U S A 107(14):6328–6333

Pfeffer S, Zavolan M, Grasser FA, Chien M, Russo JJ, Ju J et al (2004) Identification of virus-encoded microRNAs. Science 304(5671):734–736

Pfeffer S, Sewer A, Lagos-Quintana M, Sheridan R, Sander C, Grasser FA et al (2005) Identification of microRNAs of the herpesvirus family. Nat Methods 2(4):269–276

Pollicino T, Belloni L, Raffa G, Pediconi N, Squadrito G, Raimondo G et al (2006) Hepatitis B virus replication is regulated by the acetylation status of hepatitis B virus cccDNA-bound H3 and H4 histones. Gastroenterology 130(3):823–837

Popper H, Roth L, Purcell RH, Tennant BC, Gerin JL (1987) Hepatocarcinogenicity of the woodchuck hepatitis virus. Proc Natl Acad Sci U S A 84(3):866–870

Pratt ZL, Kuzembayeva M, Sengupta S, Sugden B (2009) The microRNAs of Epstein-Barr virus are expressed at dramatically differing levels among cell lines. Virology 386(2):387–397

Qin Z, Freitas E, Sullivan R, Mohan S, Bacelieri R, Branch D et al (2010a) Upregulation of xCT by KSHV-encoded microRNAs facilitates KSHV dissemination and persistence in an environment of oxidative stress. PLoS Pathog 6(1):e1000742

Qin Z, Kearney P, Plaisance K, Parsons CH (2010b) Pivotal advance: Kaposi's sarcoma-associated herpesvirus (KSHV)-encoded microRNA specifically induce IL-6 and IL-10 secretion by macrophages and monocytes. J Leukoc Biol 87(1):25–34

Qiu L, Fan H, Jin W, Zhao B, Wang Y, Ju Y et al (2010) miR-122-induced down-regulation of HO-1 negatively affects miR-122-mediated suppression of HBV. Biochem Biophys Res Commun 398(4):771–777

Raab-Traub N (2012) Novel mechanisms of EBV-induced oncogenesis. Curr Opin Virol 2(4): 453–458

Ramakrishnan R, Donahue H, Garcia D, Tan J, Shimizu N, Rice AP et al (2011) Epstein-Barr virus BART9 miRNA modulates LMP1 levels and affects growth rate of nasal NK T cell lymphomas. PLoS One 6(11):e27271

Riley KJ, Rabinowitz GS, Yario TA, Luna JM, Darnell RB, Steitz JA (2012) EBV and human microRNAs co-target oncogenic and apoptotic viral and human genes during latency. EMBO J 31(9):2207–2221

Sachdeva M, Mo YY (2010) MicroRNA-145 suppresses cell invasion and metastasis by directly targeting mucin 1. Cancer Res 70(1):378–387

Sachdeva M, Zhu S, Wu F, Wu H, Walia V, Kumar S et al (2009) p53 represses c-Myc through induction of the tumor suppressor miR-145. Proc Natl Acad Sci U S A 106(9):3207–3212

Samols MA, Hu J, Skalsky RL, Renne R (2005) Cloning and identification of a microRNA cluster within the latency-associated region of Kaposi's sarcoma-associated herpesvirus. J Virol 79(14):9301–9305

Samols MA, Skalsky RL, Maldonado AM, Riva A, Lopez MC, Baker HV et al (2007) Identification of cellular genes targeted by KSHV-encoded microRNAs. PLoS Pathog 3(5):e65

Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM (1990) The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 63(6):1129–1136

Seeger C, Mason WS (2000) Hepatitis B virus biology. Microbiol Mol Biol Rev MMBR 64(1):51–68

Seto E, Moosmann A, Gromminger S, Walz N, Grundhoff A, Hammerschmidt W (2010) Micro RNAs of Epstein-Barr virus promote cell cycle progression and prevent apoptosis of primary human B cells. PLoS Pathog 6(8):e1001063

Shi M, Du L, Liu D, Qian L, Hu M, Yu M et al (2012) Glucocorticoid regulation of a novel HPV-E6-p53-miR-145 pathway modulates invasion and therapy resistance of cervical cancer cells. J Pathol 228(2):148–157

Shrivastava S, Petrone J, Steele R, Lauer GM, Bisceglie AM, Ray RB (2013) Upregulation of circulating miR-20a is correlated with hepatitis C virus mediated liver disease progression. Hepatology 58(3):863–871

Skalsky RL, Cullen BR (2010) Viruses, microRNAs, and host interactions. Annu Rev Microbiol 64:123–141

Skalsky RL, Samols MA, Plaisance KB, Boss IW, Riva A, Lopez MC et al (2007) Kaposi's sarcoma-associated herpesvirus encodes an ortholog of miR-155. J Virol 81(23):12836–12845
Skalsky RL, Corcoran DL, Gottwein E, Frank CL, Kang D, Hafner M et al (2012) The viral and cellular microRNA targetome in lymphoblastoid cell lines. PLoS Pathog 8(1):e1002484
Song K, Han C, Zhang J, Lu D, Dash S, Feitelson M et al (2013) Epigenetic regulation of miR-122 by PPARgamma and hepatitis B virus X protein in hepatocellular carcinoma cells. Hepatology. doi:10.1002/hep.26514. [Epub ahead of print]
Su C, Hou Z, Zhang C, Tian Z, Zhang J (2011) Ectopic expression of microRNA-155 enhances innate antiviral immunity against HBV infection in human hepatoma cells. Virol J 8:354
Suzuki HI, Yamagata K, Sugimoto K, Iwamoto T, Kato S, Miyazono K (2009) Modulation of microRNA processing by p53. Nature 460(7254):529–533
Taraboletti G, Benelli R, Borsotti P, Rusnati M, Presta M, Giavazzi R et al (1999) Thrombospondin-1 inhibits Kaposi's sarcoma (KS) cell and HIV-1 Tat-induced angiogenesis and is poorly expressed in KS lesions. J Pathol 188(1):76–81
Terradillos O, Billet O, Renard CA, Levy R, Molina T, Briand P et al (1997) The hepatitis B virus X gene potentiates c-myc-induced liver oncogenesis in transgenic mice. Oncogene 14(4):395–404
Tian Y, Yang W, Song J, Wu Y, Ni B (2013) Hepatitis B virus x protein-induced aberrant epigenetic modifications contributing to human hepatocellular carcinoma pathogenesis. Mol Cell Biol 33(15):2810–2816
Tie J, Pan Y, Zhao L, Wu K, Liu J, Sun S et al (2010) MiR-218 inhibits invasion and metastasis of gastric cancer by targeting the Robo1 receptor. PLoS Genet 6(3):e1000879
Tili E, Michaille JJ, Cimino A, Costinean S, Dumitru CD, Adair B et al (2007) Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. J Immunol 179(8):5082–5089
Tsai WC, Hsu SD, Hsu CS, Lai TC, Chen SJ, Shen R et al (2012) MicroRNA-122 plays a critical role in liver homeostasis and hepatocarcinogenesis. J Clin Invest 122(8):2884–2897
Uesugi A, Kozaki K, Tsuruta T, Furuta M, Morita K, Imoto I et al (2011) The tumor suppressive microRNA miR-218 targets the mTOR component Rictor and inhibits AKT phosphorylation in oral cancer. Cancer Res 71(17):5765–5778
Umbach JL, Cullen BR (2010) In-depth analysis of Kaposi's sarcoma-associated herpesvirus microRNA expression provides insights into the mammalian microRNA-processing machinery. J Virol 84(2):695–703
Ura S, Honda M, Yamashita T, Ueda T, Takatori H, Nishino R et al (2009) Differential microRNA expression between hepatitis B and hepatitis C leading disease progression to hepatocellular carcinoma. Hepatology 49(4):1098–1112
van der Meer AJ, Farid WR, Sonneveld MJ, de Ruiter PE, Boonstra A, van Vuuren AJ et al (2013) Sensitive detection of hepatocellular injury in chronic hepatitis C patients with circulating hepatocyte-derived microRNA-122. J Viral Hepat 20(3):158–166
Vereide DT, Seto E, Chiu YF, Hayes M, Tagawa T, Grundhoff A et al (2013) Epstein-Barr virus maintains lymphomas via its miRNAs. Oncogene. doi:10.1038/onc.2013.71. [Epub ahead of print]
Waidmann O, Bihrer V, Pleli T, Farnik H, Berger A, Zeuzem S et al (2012) Serum microRNA-122 levels in different groups of patients with chronic hepatitis B virus infection. J Viral Hepat 19(2):e58–e65
Wang B, Majumder S, Nuovo G, Kutay H, Volinia S, Patel T et al (2009a) Role of microRNA-155 at early stages of hepatocarcinogenesis induced by choline-deficient and amino acid-defined diet in C57BL/6 mice. Hepatology 50(4):1152–1161
Wang X, Wang HK, McCoy JP, Banerjee NS, Rader JS, Broker TR et al (2009b) Oncogenic HPV infection interrupts the expression of tumor-suppressive miR-34a through viral oncoprotein E6. RNA 15(4):637–647. d
Wang Y, Lu Y, Toh ST, Sung WK, Tan P, Chow P et al (2010) Lethal-7 is down-regulated by the hepatitis B virus x protein and targets signal transducer and activator of transcription 3. J Hepatol 53(1):57–66

Wang S, Qiu L, Yan X, Jin W, Wang Y, Chen L et al (2012) Loss of microRNA 122 expression in patients with hepatitis B enhances hepatitis B virus replication through cyclin G(1)-modulated P53 activity. Hepatology 55(3):730–741

Wasley A, Alter MJ (2000) Epidemiology of hepatitis C: geographic differences and temporal trends. Semin Liver Dis 20(1):1–16

Wong DK, Yuen MF, Poon RT, Yuen JC, Fung J, Lai CL (2006) Quantification of hepatitis B virus covalently closed circular DNA in patients with hepatocellular carcinoma. J Hepatol 45(4):553–559

Wu X, Wu S, Tong L, Luan T, Lin L, Lu S et al (2009) miR-122 affects the viability and apoptosis of hepatocellular carcinoma cells. Scand. J Gastroenterol 44(11):1332–1339

Xia T, O'Hara A, Araujo I, Barreto J, Carvalho E, Sapucaia JB et al (2008) EBV microRNAs in primary lymphomas and targeting of CXCL-11 by ebv-mir-BHRF1-3. Cancer Res 68(5):1436–1442

Xie X, Piao L, Bullock BN, Smith A, Su T, Zhang M et al (2013) Targeting HPV16 E6-p300 interaction reactivates p53 and inhibits the tumorigenicity of HPV-positive head and neck squamous cell carcinoma. Oncogene. doi:10.1038/onc.2013.25. [Epub ahead of print]

Xu N, Papagiannakopoulos T, Pan G, Thomson JA, Kosik KS (2009) MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells. Cell 137(4):647–658

Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z et al (2012) Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. eLife 1:e00049

Yi R, Poy MN, Stoffel M, Fuchs E (2008) A skin microRNA promotes differentiation by repressing 'stemness'. Nature 452(7184):225–229

Yu F, Yao H, Zhu P, Zhang X, Pan Q, Gong C et al (2007) let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell 131(6):1109–1123

Zhang GL, Li YX, Zheng SQ, Liu M, Li X, Tang H (2010) Suppression of hepatitis B virus replication by microRNA-199a-3p and microRNA-210. Antivir Res 88(2):169–175

Zhang X, Zhang E, Ma Z, Pei R, Jiang M, Schlaak JF et al (2011a) Modulation of hepatitis B virus replication and hepatocyte differentiation by MicroRNA-1. Hepatology 53(5):1476–1485

Zhang Y, Xie RL, Croce CM, Stein JL, Lian JB, van Wijnen AJ et al (2011b) A program of microRNAs controls osteogenic lineage progression by targeting transcription factor Runx2. Proc Natl Acad Sci U S A 108(24):9863–9868

Zheng ZM, Baker CC (2006) Papillomavirus genome structure, expression, and post-transcriptional regulation. Front Biosci 11:2286–2302

Zhou J, Yu L, Gao X, Hu J, Wang J, Dai Z et al (2011) Plasma microRNA panel to diagnose hepatitis B virus-related hepatocellular carcinoma. J Clin Oncol 29(36):4781–4788

Zhu JY, Pfuhl T, Motsch N, Barth S, Nicholls J, Grasser F et al (2009) Identification of novel Epstein-Barr virus microRNA genes from nasopharyngeal carcinomas. J Virol 83(7):3333–3341

Ziegelbauer JM, Sullivan CS, Ganem D (2009) Tandem array-based expression screens identify host mRNA targets of virus-encoded microRNAs. Nat Genet 41(1):130–134

The Journal of Antibiotics (2014), 1–3
© 2014 Japan Antibiotics Research Association All rights reserved 0021-8820/14
www.nature.com/ja

NOTE

RK-270A – C, new oxindole derivatives isolated from a microbial metabolites fraction library of *Streptomyces* sp. RK85-270

Jun-Pil Jang[1,2], Toshihiko Nogawa[3], Masakazu Uramoto[3], Akiko Okano[3], Yushi Futamura[4], Takeshi Shimizu[3], Shunji Takahashi[1,3], Jae-Hyuk Jang[2], Jong Seog Ahn[2] and Hiroyuki Osada[3]

The Journal of Antibiotics advance online publication, 15 October 2014; doi:10.1038/ja.2014.141

Three new oxindole derivatives, RK-270A (**1**), B (**2**) and C (**3**) (Figure 1), were discovered and isolated from *Streptomyces* sp. RK85-270. They had an isopropylidene group at C-3 position of an oxindole skeleton, and C-1 also had a prenyl group and belonged to a class of 6-prenylated indoles. The isolation of this type of indoles was the first example as a natural product. Their cytotoxicity and antibacterial activities were evaluated.

Secondary metabolites from microorganisms are a major source of pharmaceutical leads and therapeutic agents[1] or bioprobes in a chemical biology study.[2] To obtain such valuable metabolites efficiently we have constructed a microbial metabolites fraction library and a spectral database based on the photodiode array detector attached LC/MS analysis.[3,4] Through our methodology, we have identified several structurally unique metabolites, verticilactam,[5] spirotoamides A and B,[6] pyrrolizilactone,[7] fraquinocins I and J[8] and 6-dimethylallylindole (DMAI)-3-carbaldehyde.[9] Moreover, we recently reported the advanced metabolite database Natural Products Plot (NPPlot) and discovery of new quinomycins, RK-1355A and B by the NPPlot search.[10] These results have revealed the advantage of the fraction library for isolation of new metabolites.

The fraction library of *Streptomyces* sp. RK85-270, which was isolated from a soil sample collected in Java, Indonesia in 1985, was prepared from 30 l of culture broth following the scheme as described in the previous paper.[3–6] On screening for structurally unique secondary metabolites using the database, we identified three unknown peaks with identical UV, which showed characteristic UV absorption with around 260, 265 and 300 nm indicating an indole chromophore with extended conjugation. They also showed quasi-molecular ion peaks at 242, 276 and 271 $[M+H]^+$, respectively. The related fractions were purified by C_{18}-HPLC with acetonitrile/water isocratic elution to yield compounds **1** (1.2 mg), **2** (1.4 mg) and **3** (1.3 mg) (see Supplementary Information for physicochemical properties). We report herein the structures of these three new compounds designated RK-270A (**1**), B (**2**) and C (**3**).

Compound **1** was obtained as an orange amorphous powder, and its molecular formula was determined to be $C_{16}H_{19}NO$ by HRESIMS (*m/z* 242.1542 $[M+H]^+$, calcd for $C_{16}H_{20}NO$, 242.1545). The IR spectrum implied the presence of an amide carbonyl group (1687 and 1617 cm^{-1}). The ^1H NMR spectrum in DMSO-d_6 showed four methyl signals (δ_H 1.67 (3H, s), 1.69 (3H, s), 2.26 (3H, s) and 2.47 (3H, s)) (Table 1). Two of them (δ_H 1.67 and 1.69) suggested the presence of a prenyl group with olefin and methylene signals (δ_H 5.26 (1H, m), 3.26 (2H, d, $J=7.4$ Hz)). It also showed an exchangeable NH proton (δ_H 10.31 (1H, broad singlet; brs)) and three aromatic resonances with AB-X pattern (δ_H 7.40 (1H, d, $J=8.0$ Hz), 6.74 (1H, dd, $J=8.0$, 1.1 Hz) and 6.60 (1H, d, $J=1.1$ Hz)) suggesting the presence of a trisubstituted benzene ring, which was supposed to be a part of an indole skeleton. The ^1H and ^{13}C NMR data in conjunction with the HSQC data suggested the presence of 16 carbons, comprising four methyls, one methylene, four methins and seven quaternary carbons, which included an amide carbonyl signal at δ_C 168.8. In the HSQC spectrum in DMSO-d_6, the correlation between H-10 and C-10 was observed as a very weak signal, therefore it was confirmed by HSQC spectrum in $CDCl_3$. Interpretation of the 2D NMR data including DQF-COSY, HSQC and HMBC spectra led to the construction of precise structure of **1** (Figure 2). The HMBC correlations from NH signal to C-3 and C-7a, from H-4 to C-3, C-6 and C-7a, from H-5 to C-3a and C-7 and from H-7 to C-3a constructed an oxindole skeleton and substitutions at C-3 and C-6 positions in the oxindole skeleton in consideration of AB-X coupling pattern in ^1H NMR spectra, ^{13}C NMR data and IR spectrum. The isopropylidene moiety and its attachment at C-3 position was confirmed by HMBC

[1]RIKEN Global Research Cluster, Wako, Saitama, Japan; [2]Chemical Biology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Ochang, Republic of Korea; [3]RIKEN Center for Sustainable Research Science, Saitama, Japan and [4]The Viral Hepatitis Research Foundation of Japan, Tokyo, Japan
Correspondence: Dr JS Ahn, Chemical Biology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 30 Yeongudanji-ro, Ochang-eup, Cheongwon-gun, Chungbuk 363-883, Republic of Korea.
E-mail: jsahn@kribb.re.kr
or Dr H Osada, RIKEN Center for Sustainable Research Science, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan.
E-mail: hisyo@riken.jp
Received 18 June 2014; revised 8 September 2014; accepted 16 September 2014

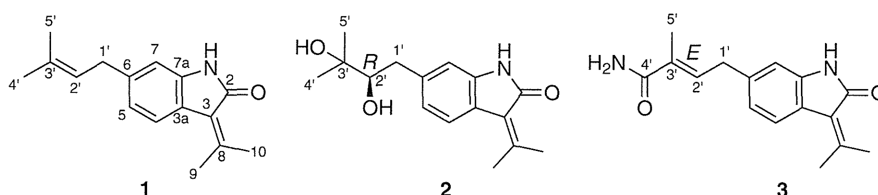

Figure 1 Structures of compounds **1**, **2** and **3**.

Table 1 NMR spectroscopic data (500 MHz) for compounds 1, 2 and 3

	1[a]		2[a]		3[a]	
Position	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1-NH	—	10.31, brs[b]	—	10.34, brs	—	10.37, brs
2	168.8	—	168.9	—	169.3[c]	—
3	122.7	—	122.8	—	123.1	—
3a	121.4	—	121.2	—	122.3	—
4	123.5	7.40, d (8.0)	123.1	7.38, d (8.0)	124.1	7.43, d (8.0)
5	120.8	6.74, dd (8.0, 1.1)	122.0	6.79, dd (8.0, 1.1)	121.5	6.79, dd (8.0, 1.2)
6	141.2	—	141.4	—	140.0	—
7	108.8	6.60, d (1.1)	110.0	6.71, d (1.1)	109.6	6.63, d (1.2)
7a	140.6	—	140.2	—	141.2	—
8	152.5	—	152.0	—	153.5	—
9	24.7	2.26, s	24.7	2.27, s	25.3	2.28, s
10	22.1	2.47, s	22.0	2.48, s	22.7	2.48, brs
1′	33.8	3.26, d (7.4)	37.6	2.88, d (13.2) 2.31, dd (13.2, 10.3)	34.5	3.43,d (7.5)
2′	123.1	5.26, m	79.1	3.27, dd (10.3, 1.2)	134.0	6.42, ddd (7.5, 7.5, 1.2)
3′	131.9	—	71.7	—	132.6	—
4′	25.5	1.69, s	24.0	1.05, s	170.5[b]	—
5′	17.7	1.67, s	26.9	1.10, s	13.3	1.82, d (1.2)
2′-OH	—	—	—	4.44, brs	—	—
3′-OH	—	—	—	4.26, brs	—	—
4′-NH_2	—	—	—	—	—	6.87, brs 7.32, brs

[a]Recorded in DMSO-d_6.
[b]brs: broad singlet.
[c]Indicated carbons showed only weak resonances in the ^{13}C NMR spectrum, but their presence and connectivity were clearly evidenced by all conducted 2D NMR experiments.

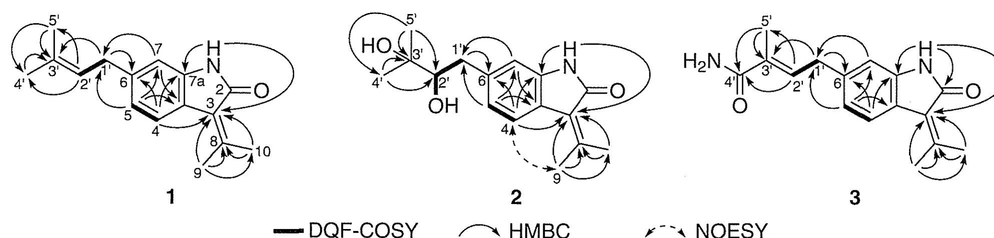

Figure 2 Key 2D NMR correlations of **1**, **2** and **3**.

correlations from both H-9 and H-10 to C-8, from H-9 to C-10, and from both H-9 and H-10 to C-3. The assignments of C-9 and C-10 signals were established by the low field chemical shift value of H-10 rather than that of H-9. The prenyl group was constructed by HMBC correlations from H-1′ to C-3′, from H-2′ to both C-4′ and C-5′, from both H-4′ and H-5′ to C-3 and from H-5′ to C-4′. The attachment of the prenyl group was established by HMBC correlation from both H-5 and H-7 to C-1′ and from H-1′ to C-6. The assignment of C-4′ and C-5′ signals were performed owing to their chemical shifts (δ_C 17.7 (C-5′) and 25.5 (C-4′)). Thus, the structure of **1** was designated as RK-270A.

The molecular formula of compound **2** was determined to be $C_{16}H_{21}NO_3$ by HRESIMS. The IR absorption at 1683 and 1622 cm^{-1} and identical UV spectrum with that of **1** suggested that **2** had the same oxindole skeleton as **1**. However, the IR spectrum showed an additional absorption at 3388 cm^{-1}, indicating the presence of a

hydroxyl group. The ^{1}H NMR spectrum in DMSO-d_6 was also similar to that of **1**, except for the disappearance of the olefin proton and appearance of two exchangeable signals at δ_H 4.44 and 4.26 as broad signals and an oxymethine signal at δ_H 3.27, which were confirmed by HSQC spectrum. The ^{13}C NMR spectrum in DMSO-d_6 showed 16 signals including identical signals for the oxindole skeleton with those of **1**. However, the olefin signals were disappeared and two oxygenated signals at δ_C 71.7 as a quaternary one and δ_C 79.1 as a methine were observed. On the basis of the above observation, **2** was supposed to be a dihydroxylated derivative of **1** at the $\Delta^{2'}$. The planner structure of **2** was established by the same manner as **1**. The assignments of C-9 and C-10 were established by their ^{1}H NMR chemical shift values and confirmed by NOESY correlation between H-4 and H-9 (Figure 2). To determine the absolute configuration for C-2′, preparation of the ester of **2** using α-methoxy-α-trifluoromethylphenylacetatic acid (MTPA) or α-methoxy-α-trifluoromethylphenylacetyl chloride (MTPACl) were carried out by application of the modified Mosher's method (see Supplementary Information for detail).[11] However, all of the approaches employed did not yield the desired product. Therefore, the optical rotation value of **2** was compared with those of (*R*)-6-(2,3-dihydroxy-3-methylbutyl)indole and (*R*)-6-(2,3-dihydroxy-3-methylbutyl)indolin-2-one.[12] Compound **2** displayed a negative optical rotation as same as the literature. Thus, the absolute configuration at C-2′ was supposed to be *R*-configuration, and the structure of **2** was designated as RK-270B.

Compound **3** had a molecular formula of $C_{16}H_{18}N_2O_2$ determined by HRESIMS. The ^{1}H and ^{13}C NMR spectra in DMSO-d_6 were similar to those of **1** for the oxindole skeleton with isopropylidene group at C-3, which was also supported by the identical UV spectrum and similar IR spectrum with those of **1**. However, one methyl signal was disappeared and two exchangeable signals assigned as NH_2 protons (δH 7.32 (1H, brs) and 6.87 (1H, brs)) were observed in the ^{1}H NMR spectrum of **3**. In addition, the ^{13}C NMR spectrum showed the additional carbonyl carbon at δ_C 170.5 as a weak signal, which was clearly observed by the HMBC correlations and confirmed by ^{13}C NMR data obtained in CD_3OD, and the missing of one methyl signal. These observation suggested that one of the methyl groups at C-3′ of **1** was replaced by an amide group. The detailed structure was determined by the same manner as **1** and **2** (Figure 2) and designated as RK-270C.

Compounds **1**, **2** and **3** were evaluated for cytotoxic activity against human cervical cancer cells (HeLa), human promyelocytic leukemia cells (HL-60), mouse temperature-sensitive cdc2 mutant cells (tsFT210) and rat kidney cells that were infected with ts25 (src^{ts}-NRK) and antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus fumigatus*, *Magnaporthe oryzae* and *Candida albicans*. Compound **1** showed moderate cytotoxicity against all of four cell lines with IC_{50} values of 6.6, 5.5, 10.9 and 15.3 $\mu g\,ml^{-1}$, respectively. Compound **1** also showed weak antifungal activity against *M. oryzae* with IC_{50} value of 8.7 $\mu g\,ml^{-1}$ In contrast, **2** and **3** did not show any effects up to 30 $\mu g\,ml^{-1}$. These results suggest that the prenyl group is important for the activities, which will be an important knowledge for future SAR study.

Three new oxindole derivatives, RK-270A (**1**), B (**2**) and C (**3**) were isolated from *Streptomyces* sp. RK85-270 based on our methodology constructing the fraction library with spectral database. They had an isopropylidene group at C-3 position and prenyl group or its related side chains at C-6 position. Even though prenylated indole derivatives are widely distributed in nature,[13] the isolation of the prenylated oxindole with the isopropylidene group at C-3 position is the first example as a natural product. We have reported the isolation of the new prenylated indole, 6-DMAI-3-carbaldehyde from *Stretomyces.* sp. SN-593 and identified the key enzyme (IptA) for the prenylation at C-6 position.[9] Recently, Satou *et al.*[14] have reported the isolation of 3-hydroxy-6-dimethylallylindolin-2-one from *Actinoplanes missouriensis* and its biosynthetic pathway. However, a gene responsible for oxindole formation still remains unsolved. In addition, conversion of **1** into **2** and **3** requires successive hydroxylation and carboxamide formation. Identification of the gene cluster of **1**–**3** is indispensable to address the mechanism of biosynthesis and future derivatization of oxindoles which might have a strong biological activity.

ACKNOWLEDGEMENTS

We thank Dr T Nakamura and Dr Y Hongo in RIKEN for the HRESIMS measurements and Ms H Aono and Ms N Morita in RIKEN for activity tests. We also thank Dr H Kusakabe in RIKEN for the collection of the strain. This work was supported in part by a Grant-in-Aid for Scientific Research (A) from the Ministry of Education, Culture, Sports and Technology of Japan, the Program for Promotion of Basic and Applied Researches for Innovations in Bio–oriented Industry and Health and Labour Sciences Research Grant. This work was also supported in part by grants of KRIBB Research Initiative Program and GRDC Program of NRF funded from Ministry of Science, ICT and Future Planning of Korea.

1 Newman D. J., Cragg G. M. Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J. Nat. Prod.* 2012; **75**: 311–335.
2 Osada, H. Chemical biology based on small molecule-protein interaction. Protein targeting with small molecules. *Chemical Biology Techniques and Applications* (ed. Osada, H.) 1–10 (Wiley, New Jersey, 2009).
3 Osada, H., Nogawa, T. Systematic isolation of microbial metabolites for natural products depository (NPDepo). *Pure Appl. Chem.* **84**, 1407–1420 (2012).
4 Kato, N., Takahashi, S., Nogawa, T., Saito, T., Osada, H. Construction of a microbial natural product library for chemical biology studies. *Curr. Opin. Chem. Biol.* **16**, 101–108 (2012).
5 Nogawa, T. *et al.* Verticilactam, a new macrolactam isolated from a microbial metabolite fraction library. *Org. Lett.* **12**, 4564–4567 (2010).
6 Nogawa, T. *et al.* Spirotoamides A and B, novel 6,6-spiroacetal polyketides isolated from a microbial metabolite fraction library. *J. Antibiot.* **65**, 123–128 (2012).
7 Nogawa, T. *et al.* Pyrrolizilactone, a new pyrrolizidinone metabolite produced by a fungus. *J. Antibiot.* **66**, 621–623 (2013).
8 Panthee, S. *et al.* Furaquinocins I and J: novel polyketide isoprenoid hybrid compounds from *Streptomyces* reveromyceticus SN-593. *J. Antibiot.* **64**, 509–513 (2011).
9 Takahashi, S. *et al.* Biochemical characterization of a novel indole prenyltransferase from *Streptomyces* sp. SN-593. *J. Bacteriol.* **192**, 2839–2851 (2010).
10 Lim, C.-L. *et al.* RK-1355A and B, novel quinomycin derivatives isolated from a microbial metabolites fraction library based on NPPlot screening. *J. Antibiot.* **67**, 323–329 (2014).
11 Ohtani, I., Kusumi, T., Kashman, Y., Kakisawa, H. High-field FT NMR application of Mosher's method. The absolute configurations of marine terpenoids. *J. Am. Chem. Soc.* **113**, 4092–4096 (1991).
12 Zheng, D. *et al.* Structure elucidation of four prenylindole derivatives from *Streptomyces* sp. isolated from Ailuropoda melanoleuca feces. *Magn. Reson. Chem.* **51**, 188–191 (2013).
13 Williams, R. M., Stocking, E. M., Sanz-Cervera, J. F. Biosynthesis of prenylated alkaloids derived from tryptophan. *Topics Curr. Chem.* **209**, 97–173 (2000).
14 Satou, R. *et al.* Isolation, structural elucidation and biosynthesis of 3-hydroxy-6-dimethylallylindolin-2-one, a novel prenylated indole derivative from *Actinoplanes missouriensis*. *J. Antibiot.* **67**, 231–236 (2014).

Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)

Adenovirus-Encoding Virus-Associated RNAs Suppress HDGF Gene Expression to Support Efficient Viral Replication

Saki Kondo[1], Kenji Yoshida[2], Mariko Suzuki[1], Izumu Saito[1], Yumi Kanegae[1]*

1 Laboratory of Molecular Genetics, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo, Japan, 2 Regenerative and Cellular Medicine Office, Sumitomo Dainippon Pharma Co., Ltd., Minatojima Minamimachi, Chuo-ku, Kobe, Japan

Abstract

Non-coding small RNAs are involved in many physiological responses including viral life cycles. Adenovirus-encoding small RNAs, known as virus-associated RNAs (VA RNAs), are transcribed throughout the replication process in the host cells, and their transcript levels depend on the copy numbers of the viral genome. Therefore, VA RNAs are abundant in infected cells after genome replication, i.e. during the late phase of viral infection. Their function during the late phase is the inhibition of interferon-inducible protein kinase R (PKR) activity to prevent antiviral responses; recently, mivaRNAs, the microRNAs processed from VA RNAs, have been reported to inhibit cellular gene expression. Although VA RNA transcription starts during the early phase, little is known about its function. The reason may be because much smaller amount of VA RNAs are transcribed during the early phase than the late phase. In this study, we applied replication-deficient adenovirus vectors (AdVs) and novel AdVs lacking VA RNA genes to analyze the expression changes in cellular genes mediated by VA RNAs using microarray analysis. AdVs are suitable to examine the function of VA RNAs during the early phase, since they constitutively express VA RNAs but do not replicate except in 293 cells. We found that the expression level of hepatoma-derived growth factor (HDGF) significantly decreased in response to the VA RNAs under replication-deficient condition, and this suppression was also observed during the early phase under replication-competent conditions. The suppression was independent of mivaRNA-induced downregulation, suggesting that the function of VA RNAs during the early phase differs from that during the late phase. Notably, overexpression of HDGF inhibited AdV growth. This is the first report to show the function, in part, of VA RNAs during the early phase that may be contribute to efficient viral growth.

Citation: Kondo S, Yoshida K, Suzuki M, Saito I, Kanegae Y (2014) Adenovirus-Encoding Virus-Associated RNAs Suppress HDGF Gene Expression to Support Efficient Viral Replication. PLoS ONE 9(10): e108627. doi:10.1371/journal.pone.0108627

Editor: Motoyuki Otsuka, The University of Tokyo, Japan

Received July 29, 2014; **Accepted** September 2, 2014; **Published** October 2, 2014

Copyright: © 2014 Kondo et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data except for the microarray data are within the paper and its Supporting Information files. All the data acquired by the microarray analysis were deposited in the NCBI Gene Expression Omnibus (NO. GSE58605).

Funding: This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology (http://www.jsps.go.jp/english/index.html) to S.K. and Y.K. and the Ministry of Health, Labour and Welfare (http://www.mhlw.go.jp/english/index.html) for Research on the Innovative Development and the Practical Application of New Drugs for Hepatitis B to I.S. This work was supported in part by the Program for Intractable Disease Research utilizing Disease-specific iPS Cells from JST to Y.K. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors of this manuscript have the following competing interests: K. Yoshida is employed by Dainippon Sumitomo Pharma Co., Ltd. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

* Email: kanegae@ims.u-tokyo.ac.jp

Introduction

It has become increasingly clear over the past decade that non-coding small RNAs play roles in viral life cycles at various ways [1–3]. Hepatitis C virus (HCV) is known to utilize host microRNA miR122, which is specifically expressed and highly abundant in the human liver, to support its efficient replication through its direct attachment to the HCV 5′ non-translation region; thus, miR122 is regarded as a therapeutic target for antiviral intervention [4–6]. Moreover, more than two hundred small RNAs derived from viruses have been identified. For example, Epstein-Barr virus (EBV) encodes two small RNAs, EBER-1 and EBER-2 [7–9], which modulate the interferon-mediated antiviral response [10].

Adenoviruses (Ads) encode two kinds of non-coding small-RNAs, known as virus-associated (VA) RNAs, VAI and VAII, that consist of 157–160 nucleotides (nts). After Ad infection, the transcription of VA RNAs starts at the same time as the E1A gene and lasts until the late phase. Since the transcription level of VA RNAs increases depending on the number of viral genome copies, VA RNAs in Ad-infected cells are abundant during the late phase, and this is one reason why the functional analysis of VA RNAs during the late phase has been investigated much more frequently than during the early phase.

The VA RNA I (VAI), which is expressed at a level of 10^8 copies per infected cell during the late phase [11], is required to establish efficient translation in virus-infected cells [12,13]. Moreover, it is well known that VAI inhibits anti-viral double-stranded RNA (dsRNA)-activated protein kinase (PKR). Also, VAI stabilizes ribosome-associated viral mRNAs, which could lead to enhanced levels of protein synthesis [14]. These findings have indicated that VAI plays a role in creating suitable conditions for viral growth, at

least during the late phase of infection. Recently, VA RNAs have been reported to be processed to microRNAs (mivaRNAs) via the cellular RNA-interference (RNAi) machinery, and mivaRNAs disturb cellular DNA expressions during the late phase [15]. However, it has not been investigated the function of VA RNA during the early phase, though the expression of VA RNAs starts immediately during the early phase of viral infection.

E1- and E3-deleted adenovirus vectors (AdVs), known as first-generation (FG) AdVs, have widely been used for the transient expression of transgenes in various cell types. FG AdVs lack E1A gene, an essential for viral replication; consequently, they neither express any viral gene product in target cells nor replicate except in 293 cells, which express E1A gene constitutively. However, since VA RNAs are transcribed by RNA polymerase III, their expressions are independent of E1A-mediated transactivation and they are always transcribed from AdV genome in AdV-infected cells. Therefore, FG AdVs are thought to be a suitable tool for the investigation of VA RNA function during the early phase of viral infection, since they express VA RNAs but do not replicate except in 293 cells. Moreover, these AdVs allow us to study the function of VA RNAs during both early and late phase using 293 cells. For this purpose, AdVs lacking VA RNA genes (VA-deleted AdVs) are essential as a control; however, VA-deleted AdVs have been difficult to generate and produce in quantities sufficient for practical use. Recently, we have developed a novel method for the efficient production of VA-deleted AdVs using a site-specific recombinase FLP [16]. A "pre-vector" containing the VA RNA region flanked by a pair of FRT sequences, which are target sequences for FLP recombinase, is constructed according to a commonly used method for the production of FG AdV [17]. This pre-vector, which is obtained at a high titer, is subsequently used to infect a 293 cell line that constitutively expresses humanized-FLPe [18] (293hde12) [19] so that the VA RNA region is removed from replicating viral genome. Since the excision efficiency of FLP in 293hde12 cells is high enough to remove almost all the VA RNA region from the very high number of viral genome copies, this method can be used to generate a high-titer of VA-deleted AdVs efficiently.

Here, we demonstrated the effect of VA RNAs expressed via FG AdVs on cellular gene expression by comparing the expression patterns between VA-deleted AdV- and FG AdV-infected cells using a microarray analysis. We found that VA RNAs expressed from FG AdVs disturbed the cellular gene expressions. Especially, the expression level of HDGF (hepatoma-derived growth factor; ENSG00000143321.14) was significantly decreased under the replication-deficient conditions; notably, HDGF expression started to decrease even during the early phase of infection in the 293 cells. Moreover, the overexpression of the HDGF gene inhibited viral growth in 293 cells, suggesting that the suppression of HDGF gene expression mediated by the VA RNAs was important for viral growth. This is the first report to show the function of VA RNAs during the early phase of infection.

Materials and Methods

Cells and AdVs

Human embryo kidney 293 cell line (ATCC) [20], human lung carcinoma A549 cell line (ATCC) [21], and human hepatocellular carcinoma derived HuH-7 cell line (RIKEN BRC) [22]were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). 293hde12 cell line [19], which is a 293 cell line possessing the hFLPe gene [18] (an improved version of the FLPe gene [23]), was cultured in DMEM supplemented with 10% FCS plus geneticin (0.75 mg/mL). After infection with AdVs, the cells were maintained in DMEM supplemented with 5% FCS without geneticin. For AraC (cytosine b-D-arabinofuranoside, hydrochloride: Sigma) treatment, the infected cells were maintained in DMEM supplemented with 5% FCS plus AraC (20 μg/mL).

The FG AdVs were prepared using 293 cells, which constitutively express adenoviral E1 genes and support the replication of E1-substituted AdVs. The VA-deleted AdVs except for HDGF- and GFP-expressing AdVs were prepared according to a method using 293U6VA-1 cells that constitutively express both VAI and VAII. HDGF-expressing and GFP-expressing VA-deleted AdVs were generated as described previously [16]. Briefly, an HDGF-expressing and a GFP-expressing unit under the control of the EF1α promoter was inserted into the SwaI cloning site at the authentic E1 substitution region in the pre-vector cosmid pAxdV-4FVF-w, and the pre-vectors were prepared using 293 cells. Subsequently, the pre-vectors were used to infect 293hde12 cells that constitutively express humanized FLPe recombinase [19] to excise the VA RNA region from the replicating viral genome. The VA-deleted AdVs transcribed less than 1% of the VA RNAs, compared with the FG AdVs, as confirmed using real-time PCR [16]. The VA-deleted AdVs and the FG AdVs were titrated using the methods described by Pei et al [24]. Briefly, the copy numbers of a viral genome that was successfully transduced into infected target cells were measured using qPCR (relative virus titer: rVT). This method enabled us to compare the various titers, since the transduction titer is not influenced by the growth rate of the 293 cells, even if an expressed gene product is deleterious to 293 cells.

Plasmids

The pVA41da plasmid [16] contains a DNA fragment covering all of VAI and VAII from nt position 10576–11034 of adenovirus type 5. The pBluescript SK (-) (Stratagene) was used as a control. The plasmids were transfected using Transfast (Promega). A pxEFGFP plasmid expressing GFP under the control of the EF1α promoter was used as a transfection control. Two days after the transfection of pVA41da plasmid into 293 cells, the cells were harvested and the total RNAs were extracted as described below to measure the HDGF mRNA levels using qPCR.

Microarray analysis

VA-deleted AdV (Axd12CARedE) and VA-containing FG AdV (AxCAdsRedE) were infected at an MOI (multiplicity of infection) of 0.5 to A549 cells for 24 h. We prepared triplicate samples for each of the conditions, and total RNA isolation was performed using a Qiagen RNeasy kit (Qiagen). A DNA microarray analysis using Affymetrix Gene-Chip technology was performed as described previously [25–27]. Briefly, 100 ng of total RNAs were used as a template for cDNA synthesis, and biotin-labeled cRNA was synthesized with a 3′ IVT Express Kit (Affymetrix). After generating the hybridization cocktails, hybridization to the DNA microarray (Genechip; Human Genome U133 Plus 2.0 Array; Affymetrix) [28] and fluorescent labeling were performed. The microarrays were then scanned with a GeneChip; Scanner 3000 7G System (Affymetrix). The data analysis was performed using GCOS software (Affymetrix). Signal detection and quantification were performed using the MAS5 algorithm with default settings. Global normalization was performed so that the average signal intensity of all the probe sets was equal to 100. For the clustering analysis, the signals were normalized and calculated to the individual scores, and the scores were visualized using Spotfire DecisionCite [29]. The analysis of variance among the groups was also performed using Spotfire DecisionCite and normalized data.