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<td><strong>Author(s)</strong></td>
<td>Tsang, CM; Yip, YL; Lo, KW; Deng, W; To, KF; Hau, PM; Lau, VMY; Takada, K; Lui, VWY; Lung, ML; Chen, H; Zeng, M; Middeldorp, JM; Cheung, ALM; Tsao, SW</td>
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Cyclin D1 overexpression supports stable EBV infection in nasopharyngeal epithelial cells

Chi Man Tsang a,b,1, Yim Ling Yip a,b,1, Kwok Wai Lo b, Wen Deng a, Ka Fai To c, Pok Man Hau a, Victoria Ming Yi Lau a, Kenzo Takada a, Vivian Wai Yan Lui d, Maria Li Lung a, Honglin Chen a, Musheng Zeng b, Jaap Michiel Middeldorp b, Annie Lai-Man Cheung a, and Sai Wah Tsao a,b,2

Departments of aAnatomy, bClinical Oncology, and cMicrobiology, Li Ka Shing Faculty of Medicine, University of Hong Kong, Hong Kong; dDepartment of Anatomical and Cellular Pathology, Chinese University of Hong Kong, Hong Kong; eInstitute for Genetic Medicine, Hokkaido University, Sapporo 060-0815, Japan; and fDepartment of Otolaryngology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213; *State Key Laboratory of Oncology in Southern China, Cancer Institute, Sun Yat-sen University, Guangzhou 510275, China; and *Department of Pathology, Vrije Universiteit University Medical Center, 1081HV, Amsterdam, The Netherlands

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Undifferentiated nasopharyngeal carcinomas (NPCs) are commonly present with latent EBV infection. However, events regulating EBV infection at early stages of the disease and the role of EBV in disease pathogenesis are largely undefined. Genetic alterations leading to activation of cyclin D1 signaling in premalignant nasopharyngeal epithelial (NPE) cells have been postulated to predispose cells to EBV infection. We previously reported that loss of p16, a negative regulator of cyclin D1 signaling, is a frequent feature of NPC tumors. Here, we report that early premalignant lesions of nasopharyngeal epithelium overexpress cyclin D1. Furthermore, overexpression of cyclin D1 is closely associated with EBV infection. Therefore we investigated the potential role of cyclin D1 overexpression in dysplastic NPE cells in vitro. In human telomerase reverse transcriptase-immortalized NPE cells, overexpression of cyclin D1 or a p16-resistant form of CDK4 (CDK4 R24C) suppressed differentiation. This suppression may have implications for the close association of EBV infection with undifferentiated NPC. In these in vitro models, we found that cellular growth arrest and senescence occurred in EBV-infected cell populations immediately after infection. Nevertheless, overexpression of cyclin D1 or a p16-resistant form of CDK4 or knockdown of p16 in the human telomerase reverse transcriptase-immortalized NPE cell lines could counteract the EBV-induced growth arrest and senescence. We conclude that dysregulated expression of cyclin D1 in NPE cells may contribute to NPC pathogenesis by enabling persistent infection of EBV.

Epstein-Barr virus | episome | viral persistence

EBV infection is detected in nearly all undifferentiated or poorly differentiated nasopharyngeal carcinoma (NPC) in endemic regions, such as southern China, including Hong Kong (1). Establishment of persistent infection in nasopharyngeal epithelial (NPE) cells is believed to be an early and essential step for NPC development (2, 3). However, events regulating EBV infection in premalignant NPE cells are largely undefined.

Although EBV infection in normal nasopharyngeal epithelium is uncommon, latent EBV infection could be detected ubiquitously in preinvasive lesions and is believed to play a crucial role in NPC pathogenesis (4). The detection of a single clonal EBV genome in these preinvasive lesions and NPC indicated that NPC pathogenesis involves clonal expansion of a single EBV-infected NPE cell (3, 4). EBV may be able to infect cells in premalignant nasopharyngeal epithelium, but it is likely that only specific clones with permissive cellular content can support long-term propagation of EBV. The current hypothesis is that genetic alterations or dysregulated cell-signaling pathways in premalignant NPE cells render them permissive for EBV persistence and malignant transformation (3, 5–7). We have established several primary and human telomerase reverse transcriptase (hTert)-immortalized cell lines to serve as cell models for EBV infection study (8). None of these cells was able to support stable EBV infection in prolonged culture, except for NP460hTert, which harbors a p16 deletion (8). We reported previously that allelic deletion of chromosome 9p (involving the loci of p16INK4a) is a common feature of NPC and can be detected in the rare, low-grade dysplastic lesions of nasopharyngeal epithelium before EBV infection (5, 9). Furthermore, using high-resolution comparative genomic hybridization arrays, we mapped the gain of a 5.3-Mb amplicon including the cyclin D1 locus in chromosome 11q13.1 in NPC (10). Concordant amplification and/or overexpression of cyclin D1 is a common event in primary NPC and was detected in 55 of 58 primary NPC biopsies (92%). Deletion of p16 and overexpression of cyclin D1 lead to dysregulation and activation of the cyclin D1 signaling pathway, respectively. However, it remains to be determined if these genetic alterations predispose NPE cells to persistent EBV infection.

Cyclin D1 is a key cell-cycle regulatory protein promoting G1/S transition in cells (11). It complexes with CDK4 or CDK6 to phosphorylate the retinoblastoma tumor suppressor protein, resulting in the release of the E2F transcription factor to promote cell-cycle progression (12). More importantly, ectopic expression of cyclin D1 has been shown to drive cell-cycle progression and to suppress terminal differentiation in several cellular systems, including keratinocytes, myotubes, adipocytes, and nerve cells (11, 13). An earlier in vitro study indicated that undifferentiated cells may be more permissive for latent EBV infection by supporting expression of EBNA1 (14). All these observations have implications for the close association of EBV infection with undifferentiated but not differentiated NPC.

We postulated that cyclin D1 may be involved directly in promoting cell-cycle progression and suppression of terminal differentiation of NPE cells, thus conferring a permissive cellular environment for persistent EBV infection and its propagation in NPE cells. In this study, we provide experimental evidence to support these roles of cyclin D1 in facilitating stable and latent EBV infection in premalignant NPE cells.

Results

Overexpression of Cyclin D1 Is Common in EBV-Infected Dysplastic NPE Tissues. We previously reported that overexpression of cyclin D1 is a common event in NPC and can be detected in more than 90% of NPC specimens examined (35 of 38 cases) (10). To


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examine if aberrant expression of cyclin D1 is associated with EBV infection at early stage of disease development, expression of cyclin D1 and EBV-encoded RNA (EBER) was examined in consecutive histopathological sections of six rare archival specimens of dysplastic NPE tissues by immunohistochemistry and in situ hybridization. Overexpression of cyclin D1 could be detected in all of the six cases examined. Overexpression of cyclin D1 coexisted with positive EBER staining in the epithelium of these dysplastic NPE tissues, supporting a close link between cyclin D1 overexpression and EBV infection in the early stage of NPC development (Fig. 1). In contrast, cyclin D1 was detected only in basal and suprabasal cell layers of the EBER-negative normal nasopharyngeal epithelium. Interestingly, LMP1 was expressed in one of the six dysplastic NPE tissues examined (Fig. 1). In our experience, LMP1 is rarely detected by immunocytochemistry in preneoplastic NPE tissues. The role of LMP1 in the early stage of NPC development remains to be determined.

**Overexpression of Cyclin D1 Conferred Resistance to Serum-Induced Differentiation in hTert-Immortalized NPE Cells.** EBV infection is closely associated with the histologically undifferentiated type of NPC. Overexpression of cyclin D1 is known to suppress cellular differentiation and can be detected readily in dysplastic NPE tissues (Fig. 1). We investigated a potential role of cyclin D1 overexpression or activation of the cyclin D1/CDK4 pathway in contributing to the undifferentiated property of dysplastic NPE cells using hTert-immortalized NPE cell lines derived from nonmalignant nasopharyngeal tissues (8). All these immortalized NPE cell lines have been propagated in serum-free medium for more than 100 passages and show no sign of growth cessation or differentiation. Serum is a potent agent commonly used to induce terminal differentiation in culturing epithelial cells. To examine if activation of the cyclin D1/CDK4 complex in immortalized NPE cells could increase the ability of NPE cells to resist differentiation, we overexpressed either cyclin D1 or a CDK4 mutant (CDK4R24C) in these immortalized NPE cell lines and examined their responses toward serum-induced differentiation. CDK4R24C is a dominantly active mutant of CDK4 insensitive to its inhibitor, p16. Overexpression of CDK4R24C resulted in constitutive activation of the cyclin D1/CDK4 complex in immortalized NPE cells. Although treatment with 10% (vol/vol) FBS effectively induced growth arrest in all the control hTert-immortalized NPE cell lines, cells overexpressing cyclin D1 or CDK4R24C were resistant to it (Fig. 2A). NPE cells overexpressing cyclin D1 or CDK4R24C continued to proliferate in the presence of serum (Fig. 2C). Furthermore, a terminally differentiated phenotype with enlarged and flattened cell morphology and with prominent intracellular keratin networks was observed in control NP550hTert or NP361hTert cells after treatment with serum but not after transduction of cyclin D1 or CDK4R24C (Fig. S1). In addition, the expression of involucrin, a marker for the terminal differentiation of squamous epithelium, was detected after FBS treatment in control NP550hTert cells but not in cells overexpressing cyclin D (Fig. 2B). A role for cyclin D1 overexpression and activation of the cyclin D1/CDK4 pathway in resisting differentiation in immortalized NPE cells was confirmed further by Western blots showing up-regulated expression of involucrin in the control immortalized NPE cells after FBS treatment but not in NPE cells overexpressing cyclin D1/CDK4R24C (Fig. 2C).

**Activation of the Cyclin D1 Pathway Supports Clonal Proliferation of EBV-infected Cells.** Next, we examined whether activation of the cyclin D1 pathway could support persistent EBV infection in hTert-immortalized cells (NP550hTert and NP361hTert). Overexpression of cyclin D1 and CDK4R24C and p16 silencing were achieved in NP550hTert or NP361hTert cells (Fig. 3A). We then subjected these NPE cells to EBV infection using a previously published protocol (8). The immortalized NPE cells were plated at a low cell density after EBV infection to observe the appearance of proliferative EBV-infected (GFP+) colonies. In our previous study, we had seen that the ability of EBV-infected NPE cells to form proliferative colonies is a reliable indication of stable EBV infection in NPE cells (8). Proliferative EBV-infected (GFP+) colonies were readily observed in hTert-immortalized cells after cyclin D1 or CDK4R24C transduction or p16 silencing, but not in the respective control cells (Fig. 3A). These results suggest that activation of the cyclin D1/CDK4 pathway supports stable EBV infection in NPE cells. Moreover, susceptibility to EBV infection was not altered significantly in NPE cells overexpressing cyclin D1 and CDK R24C or by knockdown of p16 (Fig. 3B), suggesting that the mechanism for supporting stable EBV infection is dependent more on cellular content than on an enhanced rate of infection.

We have established additional immortalized NPE cells using various defined genetic components. When examined for their ability to support EBV infection, only NPE cells immortalized by hTert in combination with either cyclin D1 or CDK4R24C were able to support stable EBV infection. Proliferative EBV-infected clones were readily observed in these cells but not in control NPE cells immortalized by hTert alone (Fig. 3B and Table 1). The ectopic expression of cyclin D1 and CDK4R24C in these cells also was confirmed by Western blotting analysis (Fig. S3). Cyclin D1 and CDK4R24C levels were two- or threefold higher in the HA-cyclin D1/CDK4R24C transfectants. Apparently, these incremental levels of cyclin D1 and CDK4R24C are sufficient to support clonal proliferation of EBV-infected NPE cells. These observations provide further evidence supporting the hypothesis.
that activation of the cyclin D1/CDK4 signaling pathway enables stable EBV infection in immortalized NPE cells.

EBV-Infected Induced Growth Inhibition and Senescence in NPE Cells Immortalized by hTert Alone. We then investigated the underlying cause of prohibiting stable EBV infection in NP550hTert and NP361hTert cells that were immortalized by hTert alone. The rates of loss of EBV-infected NP550hTert and NP361hTert cells after infection were monitored when the cells were passaged at 7, 14, and 21 days postinfection (DPI) with a splitting ratio of 1:3 (Fig. 4A). The percentage of EBV-infected cells in hTert-immortalized NPE cell lines dropped rapidly at each passage (Fig. 4A). In our previous study (8) we had observed that the EBV-infected NPE cells have a lower growth rate than uninfected cells. The loss of EBV-infected cells may be a result of uninfected cells outgrowing the EBV-infected cells during prolonged culture. We proceeded to examine the effects of EBV infection on growth and cellular senescence in hTert-immortalized NPE cells. The expression of p16 and p21 (markers for cell-cycle arrest) in EBV-infected NP550hTert cells was examined at 14 DPI by immunofluorescence staining (Fig. 4B). A higher percentage of EBV-infected NP550hTert cells was examined at 14 DPI by immunofluorescence staining (Fig. 4B). A higher percentage of EBV-infected NP550hTert cells expressed p16 and p21, respectively, than uninfected NP550hTert cells expressed p16 and p21. To confirm further the up-regulation of p16, we performed Western blot analysis for p16 expression in EBV-infected NP550hTert cells (Fig. 4C). Western blot analysis showed that p16 expression was up-regulated in EBV-infected NP550hTert cells and that the expression of p16 was higher in EBV-infected NP550hTert cells than in uninfected cells.

Overexpression of Cyclin D1 in NPE Cells Overrides Growth Inhibition and Cellular Senescence Induced by EBV Infection. To see if overexpression of cyclin D1 overrides the growth inhibition induced by EBV infection, we separated EBV-infected and uninfected cells (at 7 DPI) in both control and cyclin D1-overexpressing NP550hTert cells by FACs and compared their growth rates (Fig. 5A). The EBV-infected populations of the control vector NP550hTert cells failed to proliferate up to day 14 after sorting, whereas the NP550hTert cells overexpressing cyclin D1 continued to proliferate, albeit at a slower rate than uninfected cells (Fig. 5A). Using SA-β-galactosidase staining, we also showed that overexpression of cyclin D1 in NP550hTert cells suppressed the cellular senescence induced by EBV infection. EBV-induced cellular senescence is reduced almost fourfold in cells overexpressing cyclin D1 as compared with control cells. (C) Western blot analysis shows that overexpression of cyclin D1 overrides the growth inhibition induced by EBV infection and cellular senescence induced by EBV infection. EBV-induced cellular senescence is reduced almost fourfold in cells overexpressing cyclin D1 as compared with control cells.

Effects of Cyclin D1 Overexpression in Regulating Lytic and Latent EBV Gene Expression in Immortalized NPE Cells. Overexpression of cyclin D1 could suppress differentiation in immortalized NPE cells.
Fig. 3. Activation of the cyclin D1 pathway enhanced clonal proliferation of EBV-infected cells. (A) Western blots confirming the exogenous expression of cyclin D1 or CDK4\textsuperscript{R24C} (a p16-insensitive CDK4 mutant) or knockdown of p16 by shRNA in hTert-immortalized NPE cells. After infection with EBV, the immortalized NPE lines were sorted for GFP\textsuperscript{+} cells, were plated at a low cell density, and were inspected for the appearance of proliferative EBV-infected (GFP\textsuperscript{+}) colonies 21 d after sorting. Clonal proliferation was observed in all EBV-infected immortalized NPE cells overexpressing either cyclin D1 or CDK4\textsuperscript{R24C} or after p16 expression was knocked down but not in their control parental lines infected with an empty vector. The morphologies of proliferative EBV-infected colonies (GFP\textsuperscript{+}) from immortalized NPE cells expressing cyclin D1 or CDK4\textsuperscript{R24C} or with p16 down-regulation are shown here. (B) Clonal proliferation of EBV-infected cells also was observed in NPE cell lines immortalized by the combined action of hTert and either cyclin D1 or CDK4\textsuperscript{R24C} but not in NPE cells immortalized by hTert alone. Phase-contrast and fluorescent images show the formation of colonies in cell lines overexpressing cyclin D1 or CDK4\textsuperscript{R24C} but not in cell lines immortalized by hTert alone.

(Fig. 2). Previous studies have suggested that cellular differentiation could induce lytic reactivation of EBV (14, 15). Therefore, we investigated the effect of overexpressing cyclin D1 on the expression of lytic and latent genes of EBV by real-time PCR, immunocytochemistry, and Western blotting analysis. Seven days after infection, the EBV-infected control and cyclin D1-overexpressing NP550hTert cells were live-sorted by FACS and examined for transcript levels of lytic and latent genes. The EBV-infected cells overexpressing cyclin D1 had higher levels of mRNA transcripts of latent EBV genes (EBER1/2 and EBNA1) but lower transcript levels of EBV lytic genes (BZLF1, BRLF1, BMRF1, and BGLF4) than control NP550hTert cells (Fig. 6A). We further examined the expression of EBV-encoded proteins at the single-cell level using immunofluorescence staining. The
EBV-infected populations of both control and cyclin D1-overexpressing cells were purified by FACS at 3, 7, and 14 DPI and were examined for expression of LMP1, BZLF1, and viral capsid antigen p18 (VCAp18) (Fig. 6B). Expression of LMP1 and BZLF1 was observed in only a small subset (3–6% at 3 DPI) of EBV-infected cells (Fig. 6B). We did not observe a significant difference in the percentage of LMP1-expressing cells between the control and cyclin D1-overexpressing NPE. The population of BZLF1+ cells ranged from 4–6% and appeared to be lower in cells overexpressing cyclin D1 than in control cells (Fig. 6B). The number of cells expressing BZLF1 diminished progressively at 7 DPI and 14 DPI. Another lytic EBV protein, VCAp18, which is expressed at a late stage of lytic replication, was barely detectable 7 DPI and 14 DPI. Another lytic EBV protein, VCAp18, which is expressed at a late stage of lytic replication, was barely detectable in EBV-infected cells by immunocytochemistry (Fig. 6B).

Western blot analysis also was performed to compare the protein levels of LMP1 and BZLF1 in various EBV-infected cell lines. EBV-transformed B-cell lines, including Akata cells before and after lytic activation, and a lymphoblastoid cell line (LCL) were included as positive controls for detection of LMP1 and BZLF1 expression by Western blotting (Fig. 6C). Cyclin D1 overexpression in immortalized NPE cells apparently had a minimal impact on levels of LMP1 expression but appeared to down-regulate the expression of BZLF1 (Fig. 6C).

Western blot analysis revealed a gradual loss of LMP1 expression in the EBV-infected cells after several rounds of sorting for GFP+, whereas BZLF1 expression rapidly diminished and became undetectable after the first round of sorting (Fig. 6D). The Cp promoter is involved in the transcription of EBNA3C and -3A in EBV-infected B cells to bypass the G1/S checkpoint for maintaining the growth of LCL (16). The Cp promoter usually is active in latent EBV infection in epithelial cells. Using semiquantitative RT-PCR, we examined the activity of both Cp and Op in infected cells. We confirmed that the Cp promoter was inactive during EBV infection in epithelial cells, whereas Op was substantially activated in EBV-infected NPE cells, particularly at 100 DPI (Fig. 6E).

**Discussion**

**Cyclin D1 Expression Suppresses Growth Inhibition and Senescence Caused by EBV Infection.** The current hypothesis of NPC pathogenesis is that infection of EBV in premalignant nasopharyngeal lesions harboring genetic alterations facilitates and promotes malignant transformation of NPE cells (3, 5, 7). We previously reported that cyclin D1 overexpression is a common event in NPC (10). In this study, we report that cyclin D1 overexpression also is common in dysplastic NPE tissues and coexists with EBV infection (Fig. 1). The close association of EBV infection and overexpression of cyclin D1 prompted us to investigate if there is a functional relationship between these two early events in NPC pathogenesis. During differentiation, cyclin D1 activity is inhibited, and cells exit the cell cycle. In contrast overexpression of cyclin D1 promotes cell-cycle progression and perturbs cellular differentiation (11, 13). A role of cyclin D1 in suppressing responses of immortalized NPE cells to serum-induced differentiation was observed in this study (Fig. 2) and may have implications for the close association of EBV infection with undifferentiated NPC.

In this study, we observed that EBV-infected NPE cells immortalized by hTert alone failed to propagate and were lost rapidly upon serial passages (Figs. 3 and 4A). We attributed the rapid loss of EBV-infected NPE cells to the induction of growth inhibition and cell senescence caused by EBV infection. Growth inhibition of EBV-infected hTert-immortalized cells was associated with the expression of high levels of p16 and p21, positive expression of SA-β-galactosidase, and lower rates of BrdU incorporation (Figs. 4 and 5). The induction of growth arrest in EBV-infected NPE cells may be a reason for the infrequent detection of EBV in normal nasopharyngeal epithelium in vivo (2). Additional factors may underlie the failure of EBV-infected normal NPE cells to propagate in vivo. For example, EBV infection per se may activate host immune-defense mechanisms to
suppress the proliferation of infected cells through the induction of type I interferons (17). It also is common for the invading virus to shut down the host’s replication machinery to facilitate viral DNA replication (18, 19). For example, infection by influenza A virus, T-cell leukemia virus, and corona virus are all known to arrest the infected host cells at the G<sub>0</sub>/G<sub>1</sub> phase (20–22). Expression of EBV lytic genes, e.g., BGLF5, BZLF1, and BRLF1, also can induce cell-cycle arrest in infected cells (19, 23, 24).

Importantly, we observed that overexpression of cyclin D1 in NPE cells can rescue the growth inhibition and senescence phenotypes caused by EBV infection (Fig. 5). In this study, we showed that immortalized NPE cell lines can be infected by EBV at variable infection rates (Fig. S2). However, stably EBV-infected NPE cell lines could be established only in immortalized NPE cells overexpressing cyclin D1 or in NPE cells with dysregulated cyclin D1-related pathways by expressing the p16-insensitive CDK4<sup>R24C</sup> or p16 silencing (Table 1). In addition, we reported previously that stable EBV infection can be achieved in a telomerase-immortalized NPE cell line, NP460hTert, harboring the p16 deletion (25). Furthermore, the p16 gene, which is the key inhibitor of the cyclin D1/CDK4 activity, frequently is deleted or inactivated by methylation in premalignant nasopharyngeal epithelium before EBV infection (6, 26). We conclude that overexpression of cyclin D1 or aberrant activation of the cyclin D1 pathway may provide advantages supporting stable EBV infection in premalignant NPE cells.
Effects of Cyclin D1 on EBV Gene Expression in EBV-Infected NPE Cells.

We investigated the effects of cyclin D1 on the expression of representative latent and lytic EBV genes using real-time PCR. Up-regulation of EBNA1 and EBER1/2 was detected in EBV-infected hTert-immortalized NPE cells overexpressing cyclin D1 as compared with control cells (Fig. 6A). A main function of EBNA1 is to tether the EBV episome to the host chromosome to ensure proper segregation of EBV genomes during mitosis (27).

Fig. 5. Cyclin D1 overexpression rescued the hTert-immortalized NPE cells from the growth inhibition and cellular senescence induced by EBV infection. (A) Cyclin D1 overexpression was able to rescue NP550hTert cells from the growth-inhibiting effect of EBV infection. Growth curves were drawn for the sorted uninfected and infected populations of vector and cyclin D1-overexpressing cells. Means and SDs were calculated from triplicate wells. **P < 0.01; *P < 0.05 in a two-tailed Student t test. (B) Cyclin D1 overexpression in NP550hTert cells conferred resistance to the induction of cellular senescence upon EBV infection. The EBV-infected populations of both the control and cyclin D1-overexpressing NP550hTert cells were sorted by FACS. SA-β-galactosidase staining was performed, and phase-contrast images were taken at day 14 after sorting. Approximately 75% of the EBV-infected NP550hTert cells underwent senescence, whereas the EBV-infected NP550hTert cells overexpressing cyclin D1 sustained their proliferative ability over time, and only approximately 18% of cells underwent senescence. In the left images, the morphology of cells can be shown more clearly in the enlarged views. In the right images, enlarged views show perinuclear staining of SA-β-galactosidase. Means and SDs were calculated from triplicate wells. **P < 0.01; in a two-tailed Student t test. (C) The rate of DNA synthesis (indicated by BrdU incorporation) was higher in EBV-infected populations of NP550-cyclinD1-hTert cells than in EBV-infected NP550hTert cells (**P < 0.01; two-tailed Student t test). EBV-infected populations were live-sorted at 10 DPI. The cells then were allowed to incorporate BrdU for 24 h. Immunofluorescence staining was performed using an anti-BrdU antibody followed by an Alexa 555-tagged secondary antibody. For illustration purposes, white pseudocolor was chosen for both Hoechst and Alexa 555.
EBER expression has ant apoptotic and growth-stimulating effects on EBV-infected cells (28). Enhanced expression of EBNAn1 and EBER1/2 in cells overexpressing cyclin D1 may support stable EBV latent infection (29). On the one hand, cyclin D1 appears to suppress lytic EBV infection, because all the lytic genes examined, including BZLF1, BRLF1, BMLF1, and BGLF4, are generally down-regulated in EBV-infected NPE cells overexpressing cyclin D1 (Fig. 6A). Previous reports have shown a close association of epithelial differentiation and lytic EBV gene expression

We also confirmed that Cp is not activated in EBV-infected NP550hTert cells but is active in LCL cells (Fig. 6f). This result may explain why EBV can transform B cells into proliferative LCL cells but induces growth arrest in NPE cells (34). EBV infection in B cells initiates a growth/proliferative program. At the initial phase of infection, the Wp promoter of EBV is activated to transcribe the EBNA2 gene, driving the infected B cell to enter the cell cycle from the resting phase (G0) (16). EBNA2 also acts as a transcription factor that activates the viral Cp promoter to transcribe other genes, including EBNAn4A and -3A, which have been shown to repress the expression of p16 and p14 to facilitate cell-cycle progression and growth of LCL (35). However, neither the Wp nor the Cp promoter is activated during EBV infection in epithelial cells (36). Clearly, the cellular content of NPE cells is different from that of B cells and is not permissive for the transcription of EBV genes, which are known to override the cell-cycle checkpoints of infected host cells. Hence, genetic alterations, such as overexpression of cyclin D1 and inactivation of p16, which dampen the growth arrest and senescence stimuli, may play an important role in facilitating the persistence of EBV infection in NPE cells.

Several rounds of sorting of EBV-infected populations in cell lines with deregulated cyclin D1 pathways led to the establishment of stably EBV-infected cell lines (Table 1, Fig. 7A, and Figs. S4 and S5). We determined the EBV genome load in NP550hTert+ cyclinD1+EBV cells and NP361hTert+cyclinD1+EBV cells using in situ hybridization of fluorescent probes specific for EBV DNA (Fig. 7B). The high copy number of EBV in both cell lines suggests that the cells amplified the episomal virus genome after infection (36). This amplification may contribute to the persistence of EBV propagation. The examination of EBV gene expression profile revealed that all the stably infected cell lines express EBNAn1, LMP1, LMP2A, and EBER1/2, as shown by Western blots or real-time PCR (Fig. 7C and Fig. S6), indicating that EBV infection manifested type II latency in these NPE cell lines, resembling the expression pattern in NPC tumors.
In this study, we show that premalignant nasopharyngeal epithelium commonly overexpresses cyclin D1 and is closely associated with EBV infection. We further demonstrate that overexpression of cyclin D1 can suppress differentiation. EBV infection in NPE cells induces growth arrest and cellular senescence that can be suppressed by the overexpression of cyclin D1 or by dysregulation of the p16/CDK4 pathway. Overexpression of cyclin D1 or CDK4\(^{R24C}\) in immortalized NPE cells supports stable EBV infection, leading to the establishment of stable EBV-infected cell lines expressing latent EBV genes. Our study provides evidence that preexisting genetic events, notably cyclin D1 overexpression, and related molecular events support the establishment of stable EBV infection in premalignant nasopharyngeal epithelium and play a role in NPC pathogenesis.

Materials and Methods

Please refer to SI Materials and Methods for detailed descriptions of experimental procedures and reagents used in this study. The immortalized NPE cell lines were established and were maintained in a serum-free medium with growth factor supplements. Expression of hTert, cyclin D1, or CDK4\(^{R24C}\) in primary NPE cells was achieved by retrovirus infection. Immortalized cell lines with p16 knockdown were generated using lentiviral-mediated expression of shRNA.

The NPE cells were infected with EBV using a previously published protocol (8). The detailed protocol of the EBV infection and the experimental procedures of FACS analysis, live-cell sorting, SA-β-galactosidase assay, proliferation and colony formation assays of EBV-infected cells, the BrdU incorporation assay, and FISH are described in SI Materials and Methods.

Western blotting, immunofluorescence staining, real-time PCR, and semiquantitative PCR were used to assess protein or gene expression in EBV-infected cells. The probes or primers used in real-time PCR or PCR are listed in Table S1. Detailed experimental procedures are described in SI Materials and Methods.

In this study, H&E staining, cyclin D1 immunostaining, and EBER in situ hybridization were performed on consecutive sections of six biopsies of dysplastic nasopharyngeal tissue.

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