Cyclin D1 overexpression supports stable EBV infection in nasopharyngeal epithelial cells

Chi Man Tsang1, Yim Ling Yip1,2, Kwok Wai Lo3,4,5,6,7, Wen Deng4, Ka Fai To8, Pok Man Hau9, Victoria Ming Yi Lau9, Kenzo Takada3, Vivian Wai Yan Lui3, Maria Li Lung9, Honglin Chen5, Musheng Zeng9, Jaap Michiel Middeldorp9, Annie Lai-Man Cheung10, and Sai Wah Tsao1,2

Departments of 1Anatomy, 2Clinical Oncology, and 3Microbiology, Li Ka Shing Faculty of Medicine, University of Hong Kong, Hong Kong; 4Department of Anatomical and Cellular Pathology, Chinese University of Hong Kong, Hong Kong; 5Institute for Genetic Medicine, Hokkaido University, Sapporo 060-0815, Japan; 6Department of Otolaryngology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213; 7State Key Laboratory of Oncology in Southern China, Cancer Institute, Sun Yat-sen University, Guangzhou 510275, China; and 8Department of Pathology, Vrije Universiteit Medical Center, 1081HV, Amsterdam, The Netherlands

AUTHOR SUMMARY

The EBV genome has been detected in almost all, if not all, undifferentiated nasopharyngeal carcinoma (NPC) cells (1). EBV infection has been postulated to be an important etiological factor for NPC development (2). Clonal EBV genomes are present in early preinvasive dysplastic lesions and carcinomas in situ in nasopharyngeal epithelium, indicating that EBV infection is an early event in NPC development (3). The establishment of persistent EBV infection in premalignant nasopharyngeal epithelial (NPE) cells may represent a crucial step in the pathogenesis of NPC. Interestingly, EBV readily infects and propagates in B cells but not in NPE cells. Furthermore, EBV episomes in infected NPE are lost rapidly during long-term propagation in culture. At present, events regulating the replication and propagation of EBV in NPE cells are largely unknown. We previously reported the presence of genetic alterations, including the allelic deletion of chromosome 9p (which includes the p16 locus), in low-grade dysplastic lesions and histologically normal nasopharyngeal epithelium from human individuals at a high risk of developing NPC (4). Furthermore, the overexpression of cyclin D1, a protein involved in the regulation of cell-cycle progression, is common in primary NPC biopsies (detected in 35 of 38 cases) (5). The common deletion of p16, a key protein involved in inhibiting the activity of cyclin D1/CDK4 complex, in premalignant and cancerous nasopharyngeal epithelium and the frequent overexpression of cyclin D1 in NPC indicate that the dysregulation of the cyclin D1 pathway has a significant impact on the maintenance and propagation of EBV in premalignant NPC cells. It has been postulated that these genetic alterations in premalignant NPC cells provide a permissive cellular environment that supports the clonal expansion and propagation of EBV. In this study, we observed that EBV infection of telomerase-immortalized NPE cells induced cell-cycle arrest and senescence. Overexpression of cyclin D1 could suppress these growth-inhibitory effects associated with EBV infection and allow long-term propagation of EBV in infected NPE cells.

We previously reported that overexpression of cyclin D1 is closely associated with NPC (5). The relationship between cyclin D1 expression and EBV infection in dysplastic nasopharyngeal epithelium is unknown. It is difficult to find dysplastic NPE tissues, because most patients with NPC present clinically with late stages of the disease. However, for this study, we were able to retrieve six cases from our archival pathological specimens of dysplastic nasopharyngeal epithelial tissues. The overexpression of cyclin D1 was observed in all six of these cases, as was the coexisting expression of EBV-encoded RNA (EBER), which is a reliable indicator of EBV infection. This finding indicates that cyclin D1 overexpression and EBV infection are closely correlated in the early stage of NPC development.

Because EBV infection is highly associated with poorly or undifferentiated NPC, we investigated whether overexpression of cyclin D1 or the activation of the cyclin D1/CDK4 pathway might contribute to the undifferentiated property of dysplastic NPC cells by using telomerase-immortalized NPE cell lines derived from primary NPE tissues. Cyclin D1 or CDK4R24C (a p16-insensitive mutant of CDK4) was overexpressed in the telomerase-immortalized cell lines (NP550hTert and NP361hTert) and was found to resist serum-induced differentiation. This observation may have implications for the close association of EBV infection with undifferentiated and poorly differentiated NPC but not with differentiated NPC.

We then investigated the ability of EBV to infect and propagate in NP550hTert and NP361hTert cells. We observed that the immortalization of NPE cells, per se, is not sufficient to support stable EBV propagation. EBV infection readily induced the arrest of growth in these telomerase-immortalized cell lines. Examination


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

1C.M.T. and Y.L.Y. contributed equally to this work.
2To whom correspondence should be addressed. E-mail: gwtsao@hku.hk.
3See full research article on page E3473 of www.pnas.org.
4Cite this Author Summary as: PNAS 10.1073/pnas.1202637109.
of these EBV-infected immortalized cells revealed the expression of senescence-associated β-galactosidase (a cell senescence marker) and the up-regulated expression of p16 and p21 (which are proteins that arrest the cell cycle).

By overexpressing cyclin D1 and CDK4R24C or knocking down p16 in our telomerase-immortalized cell systems, we observed that the inhibitory effects of EBV infection on the growth of NPE cells could be suppressed, resulting in multiple colonies of EBV-infected NPE cells. This result indicates that genetic alterations that impair growth inhibition may be crucial for supporting EBV infection. We also examined the effect of cyclin D1 on the regulation of representative latent and lytic EBV genes. Up-regulation of EBV latent genes (including EBNA1 and EBER1/2) and down-regulation of lytic EBV genes (including BZLF1, BRLF1, BMRF1, and BGLF4) were observed in NPE cells that overexpressed cyclin D1. BZLF1-expressing cells were lost rapidly upon serial passages, indicating that cells undergoing lytic infection might not facilitate the long-term persistence of EBV.

The establishment of persistent EBV infection in premalignant NPE cells harboring genetic alterations has long been postulated to be an early and important event in the pathogenesis of NPC. As illustrated in Fig. P1, our results provide evidence that preexisting genetic alterations in premalignant NPE cells, notably the overexpression of cyclin D1 and related molecular events, support latent EBV infection and facilitate NPC development.