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Review

The other side of the coin: Leveraging Epstein–Barr virus in research and therapy

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ABSTRACT

Epstein-Barr virus is (EBV) a ubiquitous virus prevalent in 90% of the human population. Transmitted through infected saliva, EBV is the causative agent of infectious mononucleosis (IM) and is further implicated in malignancies of lymphoid and epithelial origins. In the past few decades, research efforts primarily focused on dissecting the mechanism of EBV-induced oncogenesis. Here, we present an alternate facet of the oncovirus EBV, on its applications in research and therapy. Finally, discussions on the prospective utilization of EBV in nasopharyngeal carcinoma (NPC) diagnosis and therapy will also be presented.

Introduction

Epstein–Barr virus (EBV) is the first human tumor virus discovered 52 years ago based on the electron microscopy observation of virus particles from Burkitt's lymphoma (BL) biopsies [1]. It belongs to human herpesvirus family and infects more than 90% of the world population [2]. EBV exhibits dual tropism, infecting both B lymphocytes and epithelial cells. While infection with the virus during childhood is usually asymptomatic, EBV infection in an adolescent can manifest as infectious mononucleosis (IM) [3,4]. In immunocompetent individuals, the replication of EBV-infected B cells is kept in check by T cell immunity, driving the virus into dormancy. Nonetheless, the virus is capable of persisting in the human population by restricting expression of viral products to EBV-encoded small RNAs (EBERs) and viral microRNAs (miRNAs), establishing latency 0 profile in the memory B cell pool [5].

As an opportunistic human pathogen, EBV was proposed to be involved in B cell malignancies observed in post-transplant lymphoma and AIDS-related lymphoma when the delicate balance between the host immunity and the EBV-infected B cells is perturbed [6–8]. Unlike EBV-associated lymphoid malignancies, the link between EBV and epithelial malignancies is less clear. Although the virus transforms and immortalizes B cell upon infection, the virus does not readily infect epithelial cells, putting forth the contribution of host genetics and environmental factors in EBV-associated NPC. Therefore, it is believed that EBV establishes latency aberrantly in epithelial cells that have already undergone pre-malignant genetic changes [9] as an essential initiation step in the development of NPC. In this article, we will discuss the utility of targeting EBV gene products, the viral episome, and whole virus for research, screening, diagnostic, and future treatment of NPC (Fig. 1).

EBV gene product-ZEBRA

EBV ZEBRA protein (product of gene BZLF1) is a basic leucine zipper transcriptional activator required for latent to lytic reactivation [10,11]. Apart from its intrinsic function in activating EBV lytic cycle, ZEBRA was shown recently to cross the cell membrane and accumulate in the nucleus of lymphocytes. Specifically, a minimal domain (MD11) consisting of 43 amino acids peptide within the ZEBRA protein was found to be efficient in delivering high molecular weight proteins across the lipid bilayer of the cell membrane via direct, non-endocytosis-dependent translocation [12]. As demonstrated by Marchione et al., the utilization of MD11 permitted high-efficiency (70–100%) delivery of fully biological active cargo proteins within two hours with almost no toxicity. Indeed, using MD11, apoptosis of melanoma and colorectal tumor cells can be observed upon the successful delivery of eIF3f (f subunit of the eukaryotic initiation factor 3) across the cell membrane [13]. Taken together, such EBV peptide-based delivery system presented here could represent a potentially powerful tool in cancer treatment, by facilitating the delivery of coupled therapeutics into cancer cells.

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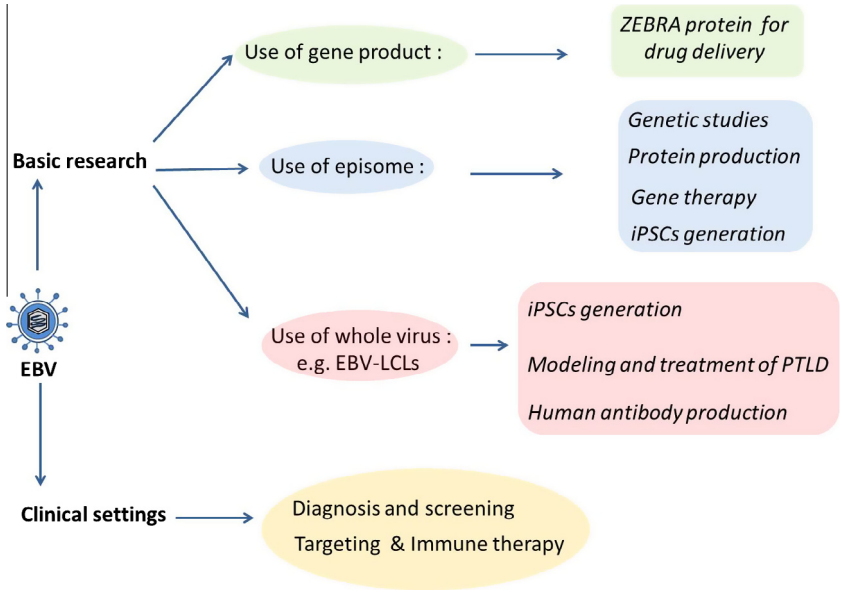

Fig. 1. Applications of EBV in basic research and clinical settings.

EBV episome

EBV episomal vectors were first described by Yates et al. in 1985 [14]. It is comprised of a latent origin of plasmid replication (oriP) and its trans-activating protein EBV nuclear antigen-1 (EBNA-1). Structurally, oriP consists of the family of repeats (FR) element and the dyad symmetry (DS) element, both containing multiple consensus sequences for EBNA-1 binding. While DS serves as the site for the initiation of replication, FR functions as an anchor point for EBNA-1 binding to ensure equal partitioning of viral episome into daughter cells. As a result, episomal vectors replicate once per cell cycle in synchrony with the host chromosomes as extra-chromosomal entities [15]. Given the non-integrative characteristic of the EBV episome and its ability to accommodate large transgene insertion, the use of EBV episomal vector had been extended to genetic studies, protein production, gene therapy, and iPSCs generation.

Employment of EBV episomes in genetic studies

Prior to the establishment of next–generation sequencing, the study of the human genome was technically challenging as the human genome is inherently large. To overcome this problem, the EBV episome was utilized as the shuttle vector to house large fragments of human genome sequences with size ranging from 60 to 330 kb [16]. The high insert capacity of EBV episomes allows the delivery of intact genomic DNA loci to achieve physiological levels of transgene expression. These constructs known as human artificial episomal chromosomes permit the preservation of the human sequences from deletion, recombination, rearrangement, and facilitated the transfer and recovery between expression systems of different organism. The use of EBV episomes had greatly expedited the process of physical mapping and functional identification of human genes.

Employment of EBV episomes for mammalian protein production

In the production of recombinant protein, a stable cell line capable of chromosomal expression of the transgene is ideal but rare [17]. While the use of viral vectors such as retrovirus, adenovirus, and baculovirus can ensure stable expression, these viruses have to be modified to prevent uncontrolled replication in case of accidental release. Apart from the laborious and technical challenges in generating these recombinant viruses, random integration of the transgene into the host chromosome with the use of these recombinant viruses can confound transgene expression and impact recombinant protein production.

In this regard, the use of EBV episome can circumvent the erratic transgene expression and recombinant protein production as the result of random integration. Episomal vectors persist in multiple copies per cell, resulting in amplification of transgene and higher protein expression in a relatively short period of time. Indeed, rapid and high transgene expression had been achieved with the use of EBV episomes in the absence of drug selection [18–20]. Coherent with the excellent capacity in accommodating large transgene, the expression of 185 kb human β-globing transgene had been reported with the stable and sustainable expression for 3 months [20]. Even though high transgene expression can also be achieved with the use of plasmid-based vectors with constitutive promoters such as SV40, CMV, and EF1 promoters, sustainability of the plasmid is always a concern as plasmid often gets diluted upon cell division.

Leveraging on the binding interaction between EBNA-1 protein and EBV episome, further improvements to achieve fast and high yield recombinant protein had been attempted. The enhancing transgene expression in EBV episomal vector is probably attributed to the binding of EBNA-1 to oriP to activate transcription of the transgene, and the oriP enabling nuclear import of transgene [21]. With the establishment of the mammalian HEK293-EBNA-1 cell line (HEK293E), where parental HEK293 cells stably express EBNA-1, the episomal expression of the transgene resulted in a threefold increase of protein yield [22–24]. As reported by Backliwal et al., the utilization of HEK293E combined with high-efficient polyethyleneimine-based transfection resulted in a remarkable efficiency of exceeding 1 g/l of recombinant protein [25]. Notebly, production of recombinant protein at the scale of 100 L with 293E adapted suspension culture had been reported by Philippe et al. [26,27] appealing to industry production for biotechnology.

Gene therapy

Gene therapy is a therapeutic approach to complement a deficient or to correct a defective gene via the introduction of a

corrective cDNA or DNA. Systems currently available utilizing viral vectors derived from retroviruses, lentiviruses, and adenoviruses pose safety concerns due to random insertion and accompanying cytopathic effects. Therefore the possibility of activating an oncogene should not be disregarded [28]. In one of most the apprehensive incident of gene therapy, the use of retrovirus to correct severe combined immune deficiency had led to the development of leukemia-like condition [29]. With its non-integrating property, EBV episomal vector provides a safer alternative to the use of viral vector. Indeed, the utility of EBV episome had been extended to the treatment of malignant and congenital disorders, accentuating its potential clinical application [30,31].

However, efficient gene delivery remains a problem. The transfection procedures for EBV episomal vectors usually include electroporation and intramuscular injection [31]. The transfection efficiency can be improved when used in combination with cationic liposomes and polyamidodamine (PAMAM) dendrimer [32]. Another approach to tackle the issue is to develop hybrid vectors, in which retroviral, adenoviral or herpes viral elements are utilized to deliver EBV-based replicon owing to high robust infectivity.

Pluripotent stem cells (iPSCs) generation

Regenerative medicine is a branch of applied research which involves the replacement of defective cells, tissues or organs to reinstate its functionality. With the advent of induced iPSCs technology in 2006, embryonic stem-like cells can be directly reprogrammed from somatic cells by forcing the expression of four transcription factors (Oct-4, Sox-2, Klf-4, and c-Myc) [33]. In the early days of iPSCs research, the Yamanaka factors were efficiently delivered by engineered retroviruses or lentiviruses. Akin to the drawback as seen with the use of viral vectors in gene therapy, random viral integration warrant safety consideration, making virally-derived iPSCs unsafe for clinical application. As such, the use of integration-free EBV episome for iPSCs reprogramming is preferred and gaining popularity [34–36]. Actually, iPSCs used for the first historic human trial for macular degeneration were reprogrammed using non-integrative EBV episomal vectors [37].

EBV-immortalized lymphoblastoid B-cell lines (EBV-BLCLs)

The oncogenic potential of EBV is best illustrated by its ability to infect B cells *in vitro* and transform them into B lymphoblastoid cell line (BLCL) capable of indefinite proliferation. Given the ability of EBV to immortalize B cells, it became apparent that the generation of these B cell lines would preserve the genetic information of an individual. As BLCLs can be cultured continuously, they provide an unlimited source of biological materials for genetic, epidemiologic and pedigree studies [38]. In addition, these cells can be thawed and frozen at users' convenience, enabling experimental observations to be made with more reliability, and at the same time minimizing intra-sample variability.

Apart from the use of EBV episome for the iPSCs generation as mentioned earlier, the other aspect of EBV involvement in iPSCs generation is the use of EBV-BLCL as starting cell type. As reported by Rajesh et al., iPSCs can be successfully reprogrammed from BLCL with the additional of episomal vectors containing Oct3/4, Sox2, NANOG and SV40-T. Intriguingly, with merely 0.5 ml of peripheral blood, BLCL can be generated and reprogrammed into iPSCs capable of differentiating into hematopoietic, neural, cardiac, and hepatocyte-like lineages. More importantly, EBV genes were not detectable at the transcriptional and protein levels in the resultant BLCL-iPSCs after 25 passages [36]. Using this methodology, virus-free iPSCs can be obtained, making an appeal to future clinical applications. The inherent plasticity of B cells, their receptivity to oriP/EBNA-1 plasmids, ease of generating BLCLs, and availability of banked BLCL collections make EBV-BLCL the ideal sources of somatic cells for iPSC generation.

Post-transplant lymphoproliferative disorder (PTLD) is a condition whereby the immunosuppression of graft recipient resulted in the inability of T cells to control the proliferation of the EBV-infected B cells. As BLCL expresses latency III profile, they are the *in vitro* counterparts of the EBV-infected B cells in PTLD patients. [39]. Specifically, EBV-BLCL is injected into immunodeficient mice to simulate PTLD patient with B cell lymphoproliferation, and such mouse xenograft is classically used as a model for the assessment of potential therapeutics.

The other utility of EBV-BLCL in the treatment of EBV-associated malignancies entails its use in stimulating EBV-specific cytotoxic T lymphocytes (CTLs). In this treatment modality, allogeneic EBV-specific CTLs are harvested from donors and incubated with irradiated BLCL *in vitro* to stimulate the expansion of EBV-specific CTLs. These EBV-specific CTLs are cultured and expanded *in vitro* prior to their administration into PTLD and NPC patients as a form of adoptive cell transfer therapy [40–42].

With the advent of using adoptive EBV-specific CTL therapy to treat PTLD, BLCL is also used to stimulate the proliferation of these EBV-specific CTLs prior to injection into the patient [43]. Taken together, the use of BLCL is relevant for the modeling and treatment of PTLD.

Antibody is the second largest class of drugs following vaccine. The use of EBV-BLCLs had also been extended to the sphere of antibody discovery and engineering. As therapeutic antibodies entering the clinical trials mostly are either from phage display or humanized in mice [44], there is a risk of immunogenicity. Therefore, ideal monoclonal antibodies (mAbs) for clinical applications should be the natural antibodies produced in the human body as a result of the *in vivo* immune response. In this regards, a fully human-derived antibody can be obtained from the screening of individuals ('responder') who are naturally exposed and making specific humoral responses to the disease, with subsequent selection of the antigen-specific B cells followed by immortalization with EBV. Using this approach, many fully human mAbs have been isolated thus far targeting dengue virus, SARS coronavirus, H5N1 influenza, HCMV and HIV-1 and plasmodia [45–50].

An innovative fully human antibody platform is involved with the screening of individuals who naturally exposed from endemic regions for antigen-specific reactivity, targeted selection of antigen-specific B cells (e.g., by flow cytometric sorting) followed by subsequent EBV transformation, rescue of the antibody V region sequences and recombinant protein production in CHO cells or plant cells. The key technology involving is to take the advantage of EBV transforming ability to immortalize immune antibody-secreting human B-lymphocytes. The poor EBV infectivity and cloning efficiency used to be a bottleneck, but now can be improved by combining CpG activation of Toll-like Receptor-9 (TLR9) and irradiated allogeneic mononuclear cells as feeder cells [51].

EBV-based NPC screening and diagnosis

Histopathological examination of nasopharyngeal biopsies and fine needle aspiration (FNAC) of neck lumps are the gold standard for NPC diagnosis. The detection of EBV DNA (by PCR) and RNA (EBER-ish) can help when histopathological examination fails in clinically doubtful cases such as occult primaries, submucosal disease and FNAC due to low accuracy. Radiotherapy has been proved to be the most effective modality of treatment if the disease is diagnosed at an early stage, with a 5-year survival probability up to 90%. Unfortunately, 75–90% of patients with NPC already have

developed local or regional spread upon diagnosis [52]. Therefore, screening for early disease in asymptomatic individuals potentially may improve NPC treatment outcomes. In endemic regions, the presence of EBV will highly suggest NPC. Since the high association with EBV infection, antibodies specific for EBV capsid antigen (IgA/VCA), early antigen (IgA/EA) and plasma DNA have been extensively studied for the screening of NPC. Data from both endemic and non-endemic regions demonstrate plasma DNA can be used for screening, diagnosis, staging, follow-up and prognostication [53–56]. Chan and Wong [53] recently studied 1318 healthy volunteers and demonstrated that plasma EBV DNA analysis is useful for early detection of NPC before it is clinically evident. Shao et al. [57] proved that plasma EBV DNA detection is a more sensitive and specific marker than the serum IgA/VCA titer for the diagnosis and monitoring of patients with NPC. Besides plasma antibody and DNA, a circulating EBV miR-BART7 and miR-BART13 [58] were shown to serve as potentially new serological biomarkers for diagnosis and prediction of treatment efficacy of NPC. In a very recent study, Zheng et al. demonstrated EBV-encoded mir-bart1-5p detection via a less invasive nasopharyngeal brush sampling can diagnose early-stage NPC with 93.5% sensitivity and 100% specificity [59]. However, all these miRNA detections need large patient cohorts across multiple centers for confirmation.

EBV-targeted therapy and immunotherapy

Successful strategies for targeting tumor cells depend on clear differences between tumor cells and normal cells. The consistent presence of EBV in every cell of undifferentiated NPC provides a unique opportunity to target the virus itself. In endemic region, EBV is exclusively found in NPC tumor cells but not in normal cells [60]. EBV-targeted therapy and immunotherapy have been recently reviewed in detail by many researchers [61,62] mainly including (1) inhibit the EBV transforming proteins (EBNA-1, LMP1 and LMP2) using antisense RNA, small interfering RNA (siRNA) and ribozyme; (2) induce loss of the EBV episome, e.g. using Hydroxyurea; (3) trigger the lytic form of EBV replication in tumor cells by expressing the IE proteins (Zta or Rta) or using radiation, chemo drugs, demethylation and histone acetylation agents, such as 5-azacytidine, Gemcitabine, HDAC inhibitors, phorbol esters and sodium butyrate. As EBV lytic replication promotes viral antigen exposure and provokes a strong host immune response, the use in conjunction with antiviral prodrug such as ganciclovir further enhances the killing of virus and tumor cells; (4) adopt T cell immune therapy by infusion *ex vivo* expanded of autologous EBV-specific CTLs from peripheral blood mononuclear cells (PBMCs) [40,41,63,64], (5) active immunotherapy by vaccinating with LMP2 pulsed autologous monocyte-derived dendritic cells or viral vector loading with EBV peptides against LMP1, LMP2 and EBNA-1 [65,66].

EBV-targeted therapy and immunotherapy represent novel therapies that have currently become a center of interest in research and development of NPC treatment. EBV-targeting therapies have so far no clinical breakthrough yet, although cytolytic virus activation (CLVA) therapy in 3 patients with end-stage NPC showed biological effect, tolerability and moderate safety [67]. Currently, immune-based strategies represent options with the most clinical benefit [68]. While these treatments advancing, knowledge to develop new therapies is emerging. In a genome-wide mutation profiling, we find that NPC has a relatively low level of genomic alteration as compared to other cancers. Therese findings suggest that epigenetic alteration possibly is an oncogenic feature of EBV infection in neoplasia [69]. Hence targeting dysregulated epigenetic regulators might be candidate targets for NPC cancer therapy [70,71]. Recently the prokaryotic type II CRISPR/Cas9 system has been adapted for genetic engineering in mammalian cells, allowing researchers to edit the genomes with unprecedented speed and precision. This highly precise and versatile system has been used in cancer modeling, cancer genomics functional analyses and cancer drug targets screening [72–74]. In a proof of concept study, Kanda et al. found that CRISPR/Cas9-mediated cleavage of EBV episomal DNA enabled cloning of disease-associated viral strains with unprecedented efficiency. Two gastric cancer cell-derived novel EBV strains were cloned [75]. The Same technology can accelerate the discovery new EBV strain variants associated with NPC, which will provide important clues about the mechanisms of EBV-mediated epithelial carcinogenesis. In a study of BL patient-derived cells, the clearance of latent EBV genomes using CRISPR-Cas9 leads to proliferation arrest and apoptosis in EBV-infected cells, with no observed cytotoxicity to uninfected cells [76]. The feasibility of CRISPR/Cas9-mediated editing of the EBV genome (EBNA-1, EBNA-3C, LMP1, and BART) has also been demonstrated in cultured NPC cell lines [77]. The dormant viral genome during latent infection provides few therapeutic targets other than itself for antiviral drug development. Thus, this strategy may lead to a generalized approach in curing latent viral infections, although many hurdles remain before this approach could be used in the clinic, such as methods of safe and efficient delivery.

Conclusion remark and perspective

The rapid expansion of knowledge in the field of EBV biology in the past decades of research had empowered the utility of EBV in domains of gene therapy, regenerative medicine (generation of clinical-grade iPSCs) and antibody discovery. With growing evidence on EBV-induced oncogenesis in both lymphoid and epithelial malignancies, the role which EBV plays is not merely associative. Indeed, emphasis on the role which EBV plays can be observed from the increasing development of immunotherapeutic and EBV-targeted therapeutic. In particularly, clinical trials using EBV-CTLs to treat NPC and lymphoma had demonstrated promising results [63,78]. As our understanding continues to grow, application of labelled-EBV RNA/DNA and protein for tracking cancer cells, monitoring metastasis or defining resection margin for surgical procedures can be envisioned.

Conflict of interest statement

The authors have no other funding, financial relationships, or conflicts of interest to disclose.

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