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Quantitative multi-target RNA profiling in Epstein-Barr virus infected tumor cells

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Epstein-Barr virus (EBV) is etiologically linked to multiple acute, chronic and malignant diseases. Detection of EBV-RNA transcripts in tissues or biofluids besides EBV-DNA can help in diagnosing EBV related syndromes. Sensitive EBV transcription profiling yields new insights on its pathogenic role and may be useful for monitoring virus targeted therapy. Here we describe a multi-gene quantitative RT-PCR profiling method that simultaneously detects a broad spectrum (n = 16) of crucial latent and lytic EBV transcripts. These transcripts include (but are not restricted to), EBNA1, EBNA2, LMP1, LMP2, BARTs, EBER1, BARF1 and ZEBRA, Rta, BGLF4 (PK), BXLF1 (TK) and BFRF3 (VCAp18) all of which have been implicated in EBV-driven oncogenesis and viral replication. With this method we determine the amount of RNA copies per infected (tumor) cell in bulk populations of various origin. While we confirm the expected RNA profiles within classic EBV latency programs, this sensitive quantitative approach revealed the presence of rare cells undergoing lytic replication. Inducing lytic replication in EBV tumor cells supports apoptosis and is considered as therapeutic approach to treat EBV-driven malignancies. This sensitive multi-primed quantitative RT-PCR approach can provide broader understanding of transcriptional activity in latent and lytic EBV infection and is suitable for monitoring virus-specific therapy responses in patients with EBV associated cancers.

1. Introduction

Epstein-Barr virus (EBV) is a human γ1-herpesvirus 4 (HHV4) with tropism for lymphoid and epithelial cells, as reflected in the nature of EBV associated autoimmune diseases and distinct malignancies, including Burkitt's and various (non-)Hodgkin's lymphomas, post-transplant lymphoproliferative disease and HIV-associated lymphomas, as well as gastric and nasopharyngeal carcinomas. Although the viral genome potentially encodes more than 80 proteins, EBV has a highly restricted viral gene expression during latency and in tumor cells (Thorley-Lawson et al., 2013; Young and Rickinson, 2004). Distinct latency programs are used by EBV for driving initial transformation of newly infected B-cell (latency type III), mimicking the germinal center reaction (latency type II), and allowing "normal" viral persistence in resting memory B cells (latency type 0), the latter showing only non-coding RNA expression. During cell division of memory B-cells, EBNA 1 (latency type I) is expressed in addition to the non-coding RNAs to maintain the viral genome (Hochberg et al., 2004). However when EBV-carrying B-cells are activated and differentiate into plasma cells viral lytic cycle gene expression is triggered (Hochberg et al., 2004; Thorley-Lawson et al., 2013; Young et al., 2016). The diversity in latency programs is most clearly reflected in EBV driven malignancies and regulated by different promoters. EBV latency gene products actively contribute to viral persistence and are linked to oncogenesis by manipulating multiple cellular pathways controlling growth, differentiation, apoptosis and homeostasis (Frappier, 2012; Middeldorp et al., 2003). Latency type III results in expression of six nuclear proteins (EBNAs 1–6), two membrane-associated proteins (LMP1 and LMP2), non-coding EBERs (EBER1 and 2) and BARTs transcripts, the latter being precursors for up to 40 distinct viral microRNAs (Thorley-Lawson et al., 2013; Qiu et al., 2011). Since the expression of the EBNA2-6 proteins induce strong immune responses, latency type III is only observed during initial phase of primary infection and in lymphomas arising in immune compromised patients. In EBV driven (non-) Hodgkin's lymphoma, gastric and nasopharyngeal carcinomas a more restricted latency pattern

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II is observed in which LMP1 (absent in gastric carcinoma), LMP2 and BARF1 (absent in lymphoma), are expressed besides EBNA1 and non-coding EBERs and BARTs. In EBV-positive lymphoma and carcinoma tissue as well as in tumor-derived cell lines and in EBV carrying circulating memory B cells, most of the viral genome is heavily methylated and the non-methylated Q promoter drives expression of EBNA1, which is crucial for viral genome maintenance in dividing cells (Ambinder et al., 1999).

During initial B-cell infection and transformation an additional protein and 3 microRNAs are expressed that originate from the BHRF1 region. The BHRF1 open reading frame encodes a viral Bcl2 homologue (Cleary et al., 1986), which is expressed as a latent protein in growth-transformed cells in vitro, but also in some Wp-restricted Burkitt's lymphoma in vivo possibly contributing to tumorigenesis by inhibiting apoptosis (Kelly et al., 2009). Otherwise the BHRF1 encoded viral Bcl-2 protein homologue is mostly known as being expressed during the viral lytic (replicative) cycle (Foghsgaard and Jäättelä, 1997).

In EBV-transformed lymphoblastoid cell lines (LCL) EBNA1 is encoded from the 3′-end of a long precursor mRNA processed by differential splicing and expressed from either the Wp or the Cp promoter (Rogers et al., 1990). In both lymphoid and epithelial tumor tissue in vivo, and related cell lines cultured ex vivo, Wp/Cp are switched off by methylation and EBNA1 RNA transcription originates from the non-methylated Q promoter. In the lytic phase yet another promoter is used for transcription of EBNA1, the F promoter (Sample et al., 1991; Brink et al., 2001).

In EBV driven tumors viral transcription is largely limited to defined latency type (Middeldorp et al., 2003). However sporadic lytic cells may be present, which (Zhang et al., 1998; Hayes et al., 1999) according to some studies may contribute to lymphomagenesis (Ma et al., 2011). The transition from latency to the reproductive phase of EBV is tightly regulated by the "immediate early" transcription factors ZEBRA (also called Zta; BZLF1) and R (Rta; BRLF1), which are essential for inducing the lytic cascade associating with expression of the viral DNA replication machinery (Murata and Tsurumi, 2014). Newly formed non-methylated viral genomes are a template for production of late mRNAs encoding structural proteins, like viral capsid proteins (eg. VCA-p18, BFRF3) and membrane glycoproteins (gp125, gp350/220; BALF4 and BLLF1, respectively), enabling the assembly and spread of new infectious virions (Aubry et al., 2014). The viral "early" antigens include enzymes essential for nucleotide metabolism and DNA replication, involving two viral kinases, Protein Kinase (PK, BGLF4) and Thymidine Kinase (TK; BXLF1), which are used as therapeutic targets since they can convert antiviral prodrugs (eg. (val)ganciclovir) to the active tri-phosphorylated form, which subsequently blocks viral DNA replication and inhibits late mRNA synthesis (Meng et al., 2010; Moore et al., 2001; Lin and Pagano, 1986). Quantitative analysis of lytic transcripts was used in previous studies to prove the mechanism of tumor treatment by viral lytic induction and subsequent tumor cell killing by antiviral drugs (ganciclovir) (Wildeman et al., 2012; Stoker et al., 2015).

EBV transcriptome activity has been widely studied in EBV infected lymphoid and epithelial cells lines to elucidate the role of EBV in tumorgenesis, as they reflect the expression profiles in patient material derived from blood leukocytes or tissue sections of different EBV related malignancies (Bell et al., 2006; Bergallo et al., 2007; Bernasconi et al., 2006; Brooks et al., 1992; Hochberg and Thorley-Lawson, 2005; Lin et al., 2010; Tang et al., 2012; Zhang et al., 2006). However a broader use of transcriptomics was hampered by difficulties to compare studies due to a lack of standardisation relative to different cell lines (Kelly et al., 2009; Bell et al., 2006; Bernasconi et al., 2006). A first attempt for standardisation of EBV profiling was performed by using in vitro transcribed RNA as standard for each target gene (Weinberger et al., 2004). To circumvent this, more recent studies use micro array approaches (Zhang et al., 2006) and RNA-seq or Nanostring based techniques (Lin et al., 2010; Tang et al., 2012). These methods need more complex laboratory and bioinformatic skills, whereas for diagnostic use a standardised method with an easy to interpret readout would be preferred. The need for accurate validation of (therapy-induced) changes in gene transcription would require an absolute quantification method. Recently a method for absolute quantification was developed using a 48:48 dynamic array IFC assay. Quantification was performed by a single plasmid containing a single copy of all target genes (Tierney et al., 2015).

We here describe a non-biased quantitative multi-primed RT-PCR to determine the number of RNA molecules of 16 viral and one cellular gene. Absolute quantification was achieved during PCR by using a standard curve derived from a plasmid pool containing all targets. This highly accurate and standardised method enables a detailed and robust analysis of relevant RNA profiles of all EBV associated malignancies. A cross examination of commonly used EBV positive cell lines of lymphoid and epithelial origin revealed that our generated RNA profiles largely matched the known latency types. However, we found that the sensitivity of the quantitative RT-PCR here described allowed the detection of low level of lytic cells as well, confirming recent findings (Lin et al., 2010; Tang et al., 2012; Tierney et al., 2015). This method proved useful in monitoring virus-specific lytic-induction therapy responses in EBV associated malignancies (Wildeman et al., 2012; Stoker et al., 2015). Accurate RNA quantification extends our understanding of the role of EBV infection in different diseases and will be useful in monitoring EBV activity in clinical samples at diagnosis and following treatment.

2. Materials and methods

2.1. Cell culture

EBV negative cell lines included Burkitt lymphoma cell lines; BJAB, Mutu9, AK31 (kindly provided by M. Ressing), the nasopharyngeal carcinoma cell line HONE-1 and the gastric carcinoma cell line AGS. EBV positive cell lines were Burkitt's lymphoma latency type I (Akata, Daudi, P3HR1 and HH514), Burkitt's lymphoma latency type III (Raji, Jijoye and Namalwa). Well studied lymphoblastoid cell lines (LCLs) established with the B95.8 type-I EBV strain (X50-7, IB4, JY and RN) or the AG876 type-II strain (JC5) were used as well. IM1 is a spontaneous LCL line obtained from an individual with Infectious Mononucleosis. The EBV nasopharyngeal carcinoma C666.1 was kindly provided by D. Thorley-Lawson. AGS-BX1 is a gastric cell line containing the Akata recombinant strain of EBV (kindly provided by L. Hutt-Fletcher) and SNU719, a naturally derived EBV-infected gastric carcinoma cell line (purchased from Korean Cell Bank). Three spontaneous wt-LCL (IK, RI and MU) were generated by in vitro outgrowth of peripheral blood mononuclear cells from healthy individuals in the presence of 1 mM Cyclosporine-A (kindly provided by R. Khanna). We generated low passage BLCL2 and 3 by freshly culturing B-cells from 3 different Caucasian donors in the presence of EBV B95.8. All lymphoid cells were cultured in RPMI-1640 medium, supplemented with 10% FCS and 100 μg/ml penicillin, 100 IU/ml streptomycin and 1 mM glutamine (P/S/G). For the induction of the lytic cycle in HH514, cells were treated with 20 ng/ml TPA and 3 mM butyric acid at 32 °C for 24 h (Middeldorp and Herbrink, 1988). AGS-BX1 cells were cultured in Ham's F12 nutrient mixture (Lonza) supplemented with 10% FCS and P/S/G. BX1-EBV virus was maintained in the AGS background by selection with 400 μg/ml Geneticin (Gibco). C666.1 were cultured in DMEM with 10% FCS and P/S/G using bovine fibronectin (Calbiochem) coated flasks. SNU719 cells were cultured in RPMI with 10% FCS and P/S/G. EBV lytic cycle gene expression in the carcinoma