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Short Communication

Analysis of Epstein–Barr virus latent gene expression in endemic Burkitt's lymphoma and nasopharyngeal carcinoma tumour cells by using quantitative real-time PCR assays

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Studies of Epstein–Barr virus (EBV)-positive cell lines have identified several forms of virus latency, but the patterns of virus gene expression in EBV-positive tumour cells appear more variable. However, it is unclear to what extent these differences merely reflect the increased sensitivities of different detection methods. Here, the design and validation of novel real-time RT-PCR assays to quantify relative levels of EBV transcripts are described. When the new assays were used to screen a collection of endemic Burkitt's lymphoma tumours, abundant Qp-driven EBNA1 expression was found, whereas the other latent transcripts (with the exception of LMP2A) were either absent or detectable only at trace levels. Analysis of 12 nasopharyngeal carcinoma biopsies revealed significant levels of EBNA1 and LMP2A transcripts in almost every case but, in contrast to previous reports, LMP1 expression was undetectable. These new quantitative assays may help to provide a clearer picture of EBV gene expression in tumour material.

Epstein–Barr virus, a B-lymphotropic herpesvirus with growth-transforming properties, is linked to a number of human lymphoid- and epithelial-cell malignancies, including post-transplant lymphoproliferative disease (PTLD), Hodgkin's disease (HD), endemic Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC) (Rickinson & Kieff, 2001). Studies of virus-positive cell lines and EBV-associated tumour biopsies have shown that EBV can adopt one of several forms of latent infection, which are distinguished by different patterns of latent antigen expression. In lymphoblastoid cell lines (LCLs), generated by the experimental infection of resting B cells *in vitro*, and in early-onset PTLD lesions arising in immunocompromised transplant patients (Young *et al.*, 1989), EBV expresses the full spectrum of latent genes; these include two small nuclear RNAs (EBERs), the highly spliced *Bam*HI A rightward transcripts (BARTs), six nuclear antigens (EBNA1, -2, -3A, -3B, -3C and -LP) and three latent membrane proteins (LMP1, -2A and -2B) (Rickinson & Kieff, 2001). This latency III form of infection is associated with the activity of two promoters, Cp and Wp, that drive the expression of all six EBNA mRNAs (Sample *et al.*, 1986; Bodescot *et al.*, 1987; Woisetschlaeger *et al.*, 1989) and additional promoters in the *Bam*HI N region that transcribe the individual LMP genes (Fennewald *et al.*, 1984; Hudson *et al.*, 1985; Laux *et al.*, 1989). However, the situation is quite different in other virus-positive tumours, which, although positive for the EBER and BART mRNAs, show more restricted patterns of virus latent antigen expression. Thus, most BL tumours and derived BL cell lines that retain the original biopsy cell phenotype *in vitro* display a latency I form of infection characterized by expression of a single viral protein, EBNA1 (Rowe *et al.*, 1987; Gregory *et al.*, 1990). In this case, the EBNA1 mRNA is transcribed from a novel promoter Qp (Schaefer *et al.*, 1995; Nonkwelo *et al.*, 1996), whereas the Wp, Cp and LMP promoters are all silent. NPC tumour cells also express EBNA1 (from Qp), together with variable levels of the LMP proteins (Raab-Traub *et al.*, 1983; Fahraeus *et al.*, 1988; Young *et al.*, 1988; Hitt *et al.*, 1989). Despite the substantial body of evidence to support these tumour-associated forms of virus latency, other studies have suggested that BL and NPC biopsies express additional latent- and lytic-cycle antigens (Cochet *et al.*, 1993; Niedobitek *et al.*, 1995; Chang *et al.*, 1998; Tao *et al.*,

Supplementary methods, a sequence alignment of LMP1 exons 2 and 3, figures showing detection of EBV transcripts by real-time RT-PCR and conventional end-point RT-PCR and a table showing TaqMan primer/probe combinations used to detect EBV transcripts are available in JGV Online.

1998; Xue *et al.*, 2002). However, at present it remains unclear whether these latter findings indicate heterogeneity in virus antigen expression *in vivo* or merely reflect the increased sensitivity of different detection methods. On this point, many earlier studies of EBV-associated tumour cells used end-point reverse transcriptase PCR (RT-PCR) assays, often coupled to two rounds of cDNA amplification, to detect EBV transcripts. While this approach is highly sensitive, it follows that positive results can arise from just a small number of isolated cells and therefore the method cannot provide meaningful information about levels of EBV transcripts. To address this problem, the current work describes the development of a panel of quantitative TaqMan RT-PCR assays to measure EBV gene expression.

Expression of viral mRNAs was quantified by TaqMan RT-PCR using an ABI Prism 7700 sequence detection system (Applied Biosystems), using primer/probe combinations to amplify the Wp- and Cp-initiated EBNA mRNAs, YUK-spliced EBNA1 mRNA, EBNA2 mRNA, LMP1 mRNA and LMP2A mRNA detectable in latency III; Qp-initiated QUK-spliced EBNA1 mRNA present in latency I; the Fp-initiated (FQUK-spliced) mRNA and BZLF1 mRNA associated with the lytic cycle; and the BARTs (full details are given in Supplementary Table S1, available in JGV Online). Whilst most of these primer and probe sequences were designed by using the B95-8 prototype EBV sequence (Baer *et al.*, 1984), in several cases it was necessary to take into account the presence of either type- or strain-specific sequence polymorphisms that might otherwise compromise PCR amplification. Thus, in the case of EBNA2, we constructed assays for both the B95-8 (type 1) and the Ag876 (type 2) sequences (Dambaugh *et al.*, 1984). Likewise, we designed an alternative assay to detect LMP2A transcripts in Chinese isolates that have been reported to contain sequence changes in exon 2 relative to B95-8 (Busson *et al.*, 1995). Finally, in the case of the highly polymorphic LMP1 gene (Sung *et al.*, 1998; Edwards *et al.*, 1999), we chose primer sequences within conserved regions spanning the exon 2–exon 3 junction region, identified by sequence analysis of a panel of virus isolates from different geographical regions (see Supplementary Fig. S1, available in JGV Online); these primers were used in conjunction with two alternative probes specific for either the B95-8 or CAO LMP1 alleles.

In a series of preliminary experiments, we developed a cDNA synthesis protocol using a pool of 3′ gene-specific primers, such that each transcript could be assayed from an aliquot of the same cDNA. We also confirmed the specificity of each primer/probe combination by using RNA isolated from suitable EBV-negative and EBV-positive reference lines and optimized each PCR to maximize the amplification efficiency (data not shown). The quantitative nature of these new assays is immediately apparent from reconstruction experiments using known numbers of EBV-positive cells, which show linearity across the full range of cell dilutions tested (see Supplementary Fig. S2, available in JGV Online); the same amplification plots also demonstrate the high sensitivity of these assays, with all primer/probe combinations (with the exception of the assay for Qp-initiated EBNA1 mRNA) being capable of detecting a single virus-infected cell. By contrast, conventional end-point RT-PCR analyses of the same cell dilutions using established protocols (Tierney *et al.*, 1994) were frequently less sensitive and, importantly, yielded signal intensities that gave a grossly distorted impression of the relative levels of EBV gene expression (see Supplementary Fig. S3, available in JGV Online).

These new assays were then used to quantify EBV transcripts in a panel of well-characterized virus-positive B-cell lines; representative data from six LCLs, four latency I BL cell lines and two Wp-restricted BL lines are shown in Fig. 1. To allow comparison between different lines, EBV gene-expression data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels in the same sample and the normalized values were expressed relative to a suitable reference line that was defined as an arbitrary value of 1 (Applied Biosystems, 1997). These reference lines included X50-7 LCL [Wp-initiated, YUK-spliced EBNA1, EBNA2 (type 1), LMP1, LMP2A and BARTs], PS-LCL (Cp-initiated EBNA transcripts), Rael-BL (QUK-spliced EBNA1 transcripts) and Sal tr-LCL containing approximately 5 % lytic cells (Fp and BZLF1 transcripts). As seen in Fig. 1(a), all LCLs consistently expressed Cp-, EBNA2, LMP1, LMP2A and YUK-spliced EBNA1 transcripts at levels similar to those seen in the corresponding reference LCL. Wp-initiated transcripts and BARTs were also seen in every case, albeit at more variable levels. Note that the QUK-spliced EBNA1 mRNA signal detectable in several LCLs reflected a small population of lytic cells expressing FQUK-spliced transcripts from the lytic-cycle promoter Fp, as the 5′ Q and 3′ U/K primer combination detects both Fp-initiated FQUK transcripts and latent Qp-initiated QUK transcripts; in these cases, the presence of cells in the lytic cycle was confirmed by low levels of BZLF1 expression (Fig. 1a). In agreement with these RNA data, all six LCLs showed detectable expression of EBNA1, EBNA2, LMP1 and LMP2A at the protein level (Fig. 1b). Screening the standard latency I BL lines by using the same PCR assays, we found that the most abundant signals were the QUK-spliced EBNA1 transcript and the BARTs. By contrast, latency III-associated mRNAs were either completely absent or only present in trace amounts (never >1 % relative to the LCL value and in many cases $<0{\cdot}1$ %). The notable exception was LMP2A, which was consistently detected at low levels. Western blotting verified EBNA1 expression in these BL lines (Fig. 1b), but was unable to confirm the presence of LMP2A protein, either because the levels of mRNA expression were too low or the LMP2A transcripts were not translated. Fig. 1 also shows the data from two atypical BL lines, Oku-BL and Ava-BL. We have shown previously that these BL lines exhibit a novel form of infection (termed Wp-restricted latency) that is characterized by Wp-driven expression of EBNA1, -3A, -3B, -3C and -LP in the absence of EBNA2 or the LMPs (Habeshaw *et al.*, 1999; Kelly *et al.*, 2002). These earlier findings are in agreement with the results of the real-time

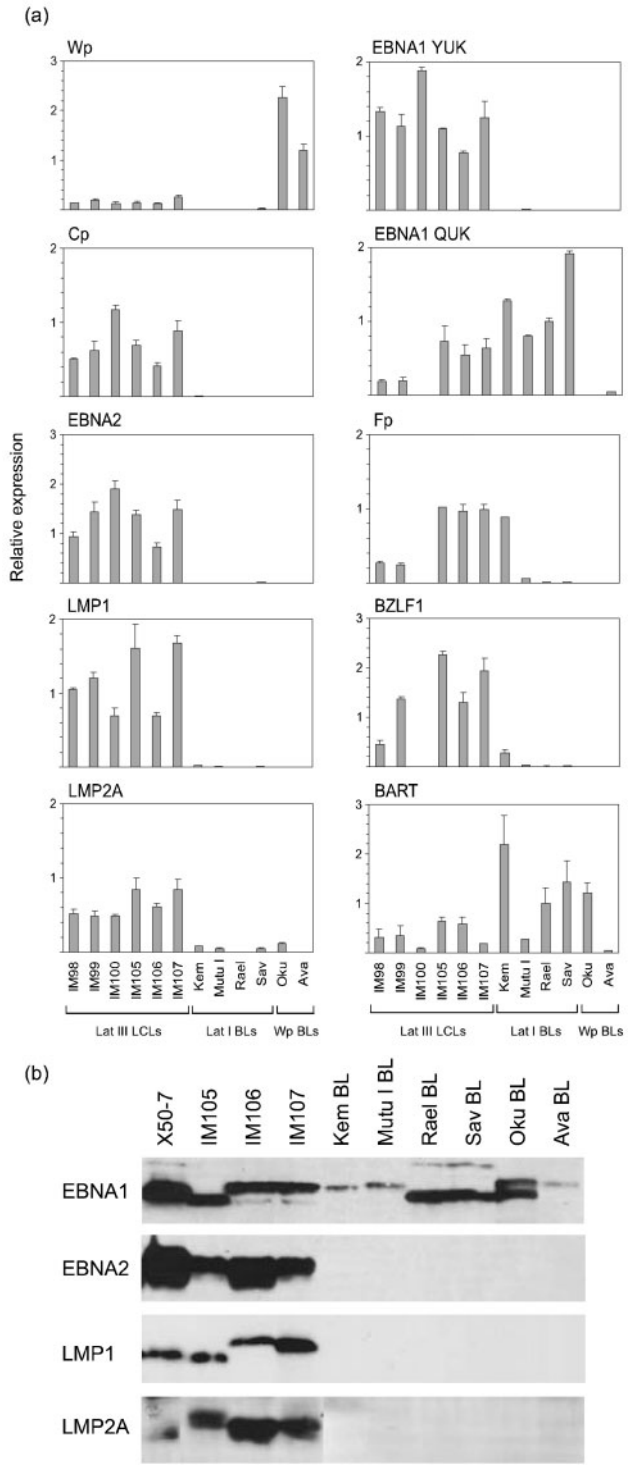

Fig. 1. EBV gene expression in LCL and BL cell lines. (a) Relative quantification of EBV transcripts by real-time RT-PCR. The histograms show the normalized value for the indicated EBV transcript expressed relative to a suitable reference cell line that has an arbitrary value of 1. Error bars indicate SD. (b) Western blot analysis of EBV latent antigen expression. Immunoblotting was carried out by using mAbs 1H4 (anti-EBNA1), PE2 (anti-EBNA2), CS1-4 (anti-LMP1) and TP14B7 (anti-LMP2). X50-7 LCL was included as a positive control.

RT-PCR analyses (Fig. 1a), which emphasize the high levels of Wp-initiated transcripts in these two BL lines.

In the next series of experiments, we asked whether the quantitative assays might provide new information about EBV gene expression in primary tumour material. In the first case, we examined a collection of endemic BL tumour biopsies from the West Nile region of Uganda (Habeshaw *et al.*, 1999) for which enough material was available to study by both RT-PCR and immunoblotting. All but one case showed a similar pattern of EBNA transcription, characterized by abundant QUK-spliced EBNA1 transcripts (at levels similar to those seen in the reference Rael BL cells) in the absence of significant Wp-, Cp- or YUK-spliced signals (Fig. 2a). The exception was the Ava-BL biopsy, which expressed high levels of Wp-initiated EBNA transcripts, a result consistent with data from the tumour-derived Ava-BL cell line. Several biopsies showed weak BZLF1 signals, but these were much weaker than in the reference line, which itself only contains 5 % lytic cells. When analysed for LMP transcripts, all BL biopsies were negative for LMP1, but showed consistent, weak signals for LMP2A. These RNA profiles are consistent with the results of immunoblotting experiments (Fig. 2b), which showed robust EBNA1 expression in 11 biopsies (EBNA1 protein was just visible in the Koko-BL sample upon long exposure), whereas there was never detectable expression of EBNA2 or LMP1. Interestingly, blotting for LMP2A revealed faint, specific bands in several tumour extracts, just visible above a non-specific band that was clearly detectable in every track (including the EBV-negative control).

We then went on to screen a series of snap-frozen NPC punch biopsies obtained from Hong Kong NPC patients (Fig. 3); in all cases, patient samples were taken following informed consent. Data are shown for 12 samples with well-preserved RNA in which we could detect robust expression of the endogenous GAPDH gene. All biopsies expressed Qp-initiated EBNA1 transcripts and the BARTs, whilst 11 of 12 samples were also positive for LMP2A mRNA (Fig. 3). By contrast, assays for Wp-, Cp- and YUK-spliced EBNA1 transcripts were always negative (data not shown), as were RT-PCR amplifications for EBNA2 mRNA in all but one case (using primer/probe combinations for both type 1 and type 2 sequences). Surprisingly, there was little, if any, evidence for LMP1 expression using assays specific for both the B95-8 LMP1 sequence (data not shown) and the Chinese CAO LMP1 sequence (Fig. 3); note that, in control experiments, we readily amplified LMP1 signals from several Chinese-derived LCLs (data not shown). Aware of the possibility that our NPC biopsies might contain rare LMP1 polymorphisms, we sequenced the tumour-derived LMP1 gene from seven biopsies for which DNA was available; we found no evidence of sequence changes in the primer or probe regions in six cases, whilst the remaining isolate contained a single nucleotide change (see Supplementary Fig. S1, available in JGV Online).

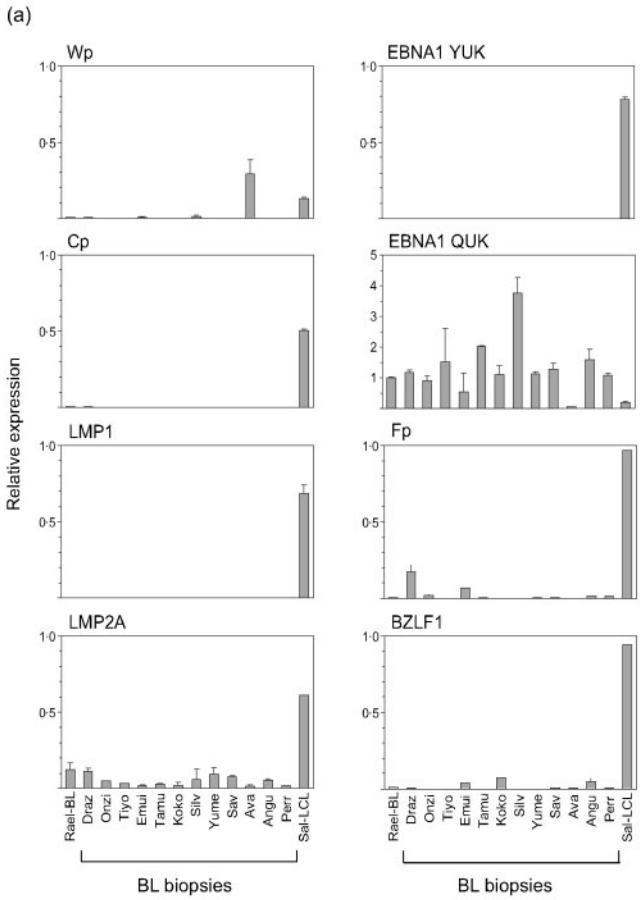

Fig. 2. EBV gene expression in BL tumour samples. (a). Relative quantification of EBV transcripts by real-time RT-PCR. The histograms show the normalized value for the indicated EBV transcript expressed relative to a suitable reference cell line that has an arbitrary value of 1. Error bars indicate SD. (b) Western blot analysis of EBV latent antigen expression. Immunoblotting was carried out by using mAbs 1H4 (anti-EBNA1), PE2 (anti-EBNA2), CS1-4 (anti-LMP1) and TP14B7 (anti-LMP2). The EBV-negative B-cell lymphoma line BJAB served as a negative control, whilst X50-7 LCL was included as a positive control.

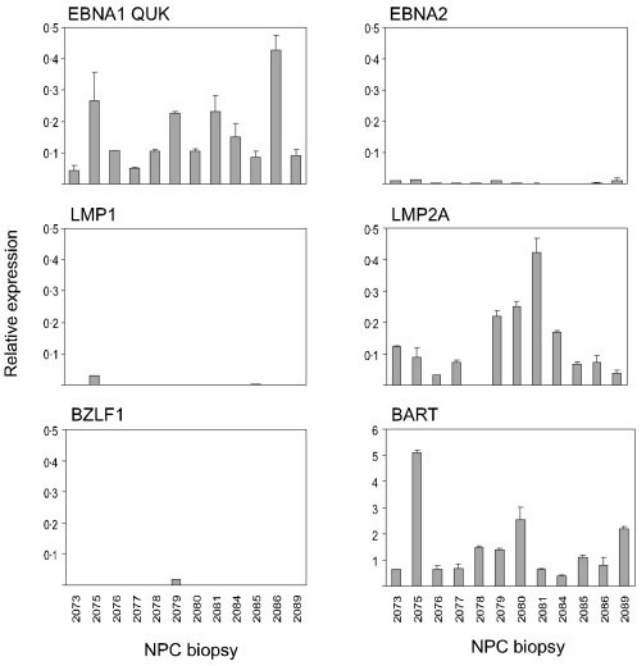

Fig. 3. EBV gene expression in NPC tumour samples determined by real-time PCR. The histograms show the normalized value for the indicated EBV transcript expressed relative to a suitable reference cell line that has an arbitrary value of 1. Error bars indicate SD.

Prompted by the need for quantitative protocols to measure EBV transcripts, the current work represents the first detailed study of EBV gene expression using TaqMan RT-PCR; this approach offers several advantages over conventional RT-PCR, including accurate quantification of mRNA levels and normalization of gene expression against an endogenous control gene (Lossos *et al.*, 2003). In this study, we have used these assays to re-examine the patterns of EBV gene expression in primary BL and NPC biopsies; in particular, we asked the following question: what is the level of expression of latent and lytic antigens in these tumours relative to the level of Qp-initiated EBNA1 transcripts?

From the analysis of early-passage BL cell lines and BL tumours (Figs 1, 2), all but the rare Wp-restricted Ava-BL showed the EBNA1-only form of infection described in previous studies (Rowe *et al.*, 1987; Gregory *et al.*, 1990). Whilst other reports have detected latency III and lytic-cycle transcripts in BL tumour cells (Tao *et al.*, 1998; Xue *et al.*, 2002), we found that such transcripts were either absent or only detectable at such low levels that they must have been derived from a small minority (usually $<0{\cdot}1\,\%$) of biopsy cells, a result consistent with single-cell studies (Niedobitek *et al.*, 1995). Thus, while we cannot exclude the possibility that there may be real differences in virus gene expression among some African BLs, our data suggest strongly that positive results in previous conventional RT-PCR studies

probably arose from rare isolated cells. However, one novel finding was the consistently low levels of LMP2A expression. Whilst LMP2A mRNA has been seen previously in BL biopsies (Tao *et al.*, 1998; Xue *et al.*, 2002), the value of the current quantitative approach is that we can estimate that these LMP2A transcripts are present at up to 10 % of LCL levels. This finding is consistent with the trace amounts of LMP2A protein detected in some biopsies by immunoblotting; unfortunately, the lack of suitable biopsy material prevented further examination of these samples at the single-cell level by immunohistochemical methods.

Screening of 12 NPC biopsies using the new assays revealed the presence of Qp-initiated EBNA1 transcripts and LMP2A transcripts in all but one case, in agreement with previous RNA studies (Brooks *et al.*, 1992; Busson *et al.*, 1992; Smith & Griffin, 1992; Chen *et al.*, 1995). Whilst the abundance of these transcripts was generally lower than in the reference lines (which in part probably reflects contamination of the biopsy with normal tissue), the similar relative strengths of these two signals suggest that both EBNA1 and LMP2A are expressed in the majority of tumour cells; the presence of significant levels of LMP2A transcripts is also in accord with a recent histological study that detected LMP2A protein in approximately 50 % of NPC tumours (Heussinger *et al.*, 2004). Interestingly, the most abundant transcripts found in our NPC biopsies were the BARTs (Hitt *et al.*, 1989; Gilligan *et al.*, 1991). Whilst the role of these transcripts in the pathogenesis of NPC remains unclear, it has recently been shown that the *Bam*HI A region of the EBV genome encodes several microRNAs, which are postulated to regulate host or viral gene expression (Pfeffer *et al.*, 2004; Cai *et al.*, 2006). Finally, one unexpected observation was the lack of detectable LMP1 mRNA in our series of NPC tumours. Whilst LMP1 transcripts have been reported in virtually all cases by using non-quantitative RT-PCR, often using two rounds of amplification (Brooks *et al.*, 1992; Chen *et al.*, 1995), LMP1 expression at the protein level is only seen in up to 60 % of cases (Fahraeus *et al.*, 1988; Young *et al.*, 1988). Further work will be needed to reconcile these differences and to test whether these observations reflect variability in EBV gene expression between different histological subtypes of NPC (Nicholls *et al.*, 1997) or whether LMP1 expression is no longer required in certain NPC tumours as cellular genetic changes substitute for its functions.

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