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Studying Activity Of Gene Of Ebv Virus In The Biopsy Of Nasopharyngeal Cancer (Npc)

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Abstract

Epstein-Barr virus (EBV) provokes the development of latent infection and is related to a number of malignant neoplasms in humans, which are considered to appear as a result of deregulation of the different stages of the virus's life cycle. A large quantity of microRNA (miRNA) for EBV has recently been described, and it was suggested that their expression may vary between different latent conditions in normal and malignant tissue. To date, however, no equipment has been used to fully and quantitatively test this idea, by defining the expression profile of miRNA EBV in primary infected tissues. We describe here a multiplex analysis of PCR with reverse transcription, which allows to profile 39 of the 40 known mature miRNA EBV.

With this approach represents an exhaustive miRNA EBV profile in nasopharyngeal primary carcinoma tumors (NPC), including estimates of miRNA copies per tumor cell. This is the first comprehensive miRNA EBV profiling in any tumor associated with EBV. Unlike previous offers, shows that miRNA from BART are present in a wide range of copies from ≤103 per cell, in both primary tumors and in widely used cell lines C666-1, from NPC. Finally, we demonstrate that expression miRNA EBV in a widely used line NPC C666-1, with certain qualifications, broadly representative of primary NPC tumors.

Keywords: Epstein-Barr virus, nasopharyngeal primary carcinoma tumors, cancer, genes, herpes virus merism.

Introduction

In Vietnam, NPC is one of the four most common cancers, and first among cancer of the head, face and neck. Because of unexposed location of tumor, it is often detected only in the late stages and misdiagnosed.

The diagnosis for NPC is mainly based on nasopharynx examination to locate the tumor and histological examinating the staging of the disease, by X-ray Hirtz, computed tomography scanner C.T. scan) and MRI.

Epstein-Barr virus. EBV is a member of c. herpes virus merism and is involved in the pathogenesis of several malignant human tumors: African forms, Burkitt lymphoma, B-cell lymphomas in AIDS patients and and other forms of immunosuppression (especially those who received immuno-suppressant therapy after organ transplants), lymphoma, like Hodgkin's lymphoma, nasopharyngeal carcinomas, some forms of stomach carcinomas, rare forms of T-cell lymphoma, and as well as NK cell lymphoma.

EBV infects B cells and possibly epithelial oral cells. EBV is attached to CD21 lymphocyte receptors, and a latent B-cell infection develops. That means there’s no replication of the virus, but B cells with latent EBV infection become immortalized and have the ability to reproduce in vitro without restriction. The molecular mechanisms of B cell proliferation are quite complex, but, as with other viral infections, it occurs with the “abduction” of several normal LMP-1, one of the EBV genes, acts as an oncogene. Expression of this protein in transgenic mice causes the development of B cell lymphoma. LMP-1 behaves the same way as one of CD40’s main active receptors that detects the signal, stimulating B-cell proliferation.

LMP-1 activates the NF kB signal pathways and JAK/ STAT, stimulates survival and proliferation of B cells, occurring autonomously in EBV-infected B-lymphocytes (without signals from T cells or other external signals). At the same time, LMP-1 prevents apoptosis, Bc1-2 activates. Thus, the virus uses activated signal pathways of normal B-cells to enlarge the pool of cells with a latent infection. Another EBV gene, EBNA-2, encodes a nuclear protein, which simulates a continuously active Notch-receptor. EBNA-2 causes the transactivation of several host genes. In addition, the EBV gene contains viral cytokine vIL-10, taken from the host’s genome. This viral cytokine may prevent activation of T cells by macrophages and monocytes and is needed for EBV-dependent transformation of B-cells. In immunologically healthy people EBV-controlled polyclonal proliferation B-cells in vivo is under control, and patient can suffer either asymptomatically, or from developing of self-regulating episode of infectious mononucleosis.

Epstein - Barr virus (EBV may be one of the causes of NPC and the presence the virus genome in nasopharyngeal epithelioma is one sign of the early stage. Serological tests are used to detect antibodies against EBV, by detecting IgA/VCA, IgA/EA, IgG/EBNA. In addition, the PCR-EBV, dot blot hybridization can detect the genome of virus in infected cells of NPC patients.

The activity of the EBV genes was often measured by he presence of mRNA-EBV, which is often increased as the disease progress. The aim of the present study to detect EBV-DNA by PCR, dot blot hybridization and activity of mRNA-EBV by RT-PCR was to high light the pathogenesis of NPC, to make early diagnosis, to predict the progress of disease and to evaluate the success of the treatment [1].

Research of cell lines, positive for the Epstein-Barr virus (EBV), detected several forms of virus latency, but patterns of viral gene expression in EBV-positive tumor cells seem to be more variable. However, it is unclear to what extent these differences simply reflect the sensitivity of different detection techniques. Here is described the development and testing of new RT-PCR analyses in real time to quantify the relative levels of EBV transcripts.

When the new tests were used for screening the collection of Berkitt-endemic lymphoma tumors, extensive Qp-controlled expression of EBNA1 has been detected, while other hidden transcripts (except LMP2A) either not present or found only at trace levels. 12 biopts of nasopharyngeal carcinoma have been identified, a significant level of EBNA1 and LMP2A transcripts in almost every case, but unlike previous reports, no LMP1 expression was detected. These new quantitative analyses can help provide a clearer picture of the expression of the EBV gene in tumor material.

Subject, materials and methods

Epstein-Barr virus (EBV) is the prevalence of human herpes virus. It infects 90 percent of the population, usually in childhood, and it lasts for life. Chronic infection is almost always benign, but acute EBV infection causes some people the kissing disease. When B cells from the blood of infected people are cultivated, they can give rise to lymphoblastics, initiation of cells development which are latently infected with EBV and express nine latent proteins (latent period III) [2].

In vivo latency III is a temporary state, through which the cell passes on the way to the persistence in the resting B-cells of memory, where latent expression of the protein is suppressed (latency 0). EBV is also associated with several malignant tumors including B cell lymphoma (Burkitt lymphoma [BL], Hodgkin's lymphoma [HL], and immunoblastic lymphoma in immunosuppression) and carcinoma (nasopharyngeal [NPC] and gastric [GC]). Viral infection in tumors is also latent, but the infection is associated with more limited forms of latent gene expression (latency I in BL and latency II in HL and NPC). However, when passing in culture tumor cells tend to drift towards latency II.

Growth in vitro in vitro vivo-infected B or tumor cells select a latent transcription program for expression, latency III, which is not representative of primary tissue. So in the EBV area, it’s very important to check if virus gene expression patterns observe in cells lines, and whether what is observed in the primary infected material deemed reasonable.

Among latent proteins, there are a few non-translated RNA, which appear to be present in all the cells studied so far, living with EBV. These include two small RNAs, called EBER1 and EBER2, and a group of alternatively spliced RNAs from a fragment of Bamhi A known as BART [3].

The EBER function is not clear, but they can play a role in cell survival, increasing the apoptotic threshold of infected cells. BART is particularly common in EBV-associated carcinomas and encode a large number of microRNAs (miRNA).

miRNAs have a length of 19 to 24 nucleotides and regulate post-transcription gene expression, by blocking transmission or causing degradation of target mRNA. These powerful gene regulators are thought to be controlling up to one third of all genes and attract increased attention because of their role in a wide range of biological functions, including differentiation, cell growth and diseases, especially cancer. Except for EBV-mir-BART2, all miRNA generated from BART, mapped to two clusters, including one new miRNA called miR-BART22.

Four miRNA clusters, that are originated from the BHRF1 gene, have also been identified. This brings the total number of known mature miRNA EBV to 40, rapidly increasing the number and complexity potentially biologically active molecules, encoded by EBV during latent infection. However, like most miRNA currently open to the public, miRNA EBV functions are poorly understood [4].

MirBART2 has been proven to degrade DNA polymerase EBV BALF5, effectively inhibit lithic replication.

It was reported that miRNA cluster 1 BART suppress expression of viral latent membrane protein 1 (LMP1), and miRNAs from the BHRF1 area are related to virus replication and chemokine regulation CXCL11. Systematic miRNA cell profiling has become an important tool in understanding the role of these molecules in human cancer and in the development of miRNA on screen as diagnostic and prognostic indicators of cancer (20, 30). Given the strong association of EBV with human cancer, systematic quantitative profiling of miRNA EBV expression patterns in EBV-associated diseases can help to reveal their potential functions and their possible worthy as predictive and diagnostic tools. To date, miRNA EBV expression studies have used Northern blotting, which is relatively insensitive and provides a qualitative assessment of expression levels [5].

Some studies have analyzed small subsets of miRNA in primary infected cells, but the most rigorous studies used EBV-infected cell lines, to offer differential expression EBV miRNA in different backlog situations.

In particular, it was proposed that miRNA BART is strongly expressed in the latent period II, while miRNA BHRF1 is highly expressed in the latent period III. But Edwards et al. reported that miRNA EBV expression does not depend on cell type, and stressed the difficulties and inconsistencies that can occur when using cell lines infected with EBV.

Until now, these problems have not been confirmed or systematically tested through exhaustive quantitative analysis of primary infected tissue. At this point, it’s a central issue, given the well-documented and powerful influence of tissue culture on latent expression. In this study, we describe the first systematic

profiling of miRNA expression as well as quantitative measurements of their expression levels in biopsy samples and cell lines. This study confirms some of the predictions made by other researches for primary tumors and provides the most complete current description of miRNA EBV in primary samples [6].

Test subjects

31 NPC patients were diagnosed by histology in K Hospital and Department ot ENT of 103 Hospital in Hanoi.

Materials and techniques

Biopsy from tumor: Histological diagnosis and type classifications were according to WHO-1978.

Isolation of RNA was carried oud using the protocol (Acid guanidinium thiocynate phenol chlorform extraction - Chomczynski and Sacchi 1987).

Isolation of DNA was carried oud using the protocol (Rapid prep microgenomic DNA isolation, code No 27-5225-01) Amersham pharmacia biotech.

PCR (Polimerase chain reaction) detection of EBV-DNA with DNA primer pair EBNA2A, kit according to the prescribed protocol (Amersham pharmacia biotech).

Dot blot hybridization detection of EBV-DNA with DNA probe is a product of PCR of the primer pair EBNA2A. This was done using ECL kit according to the prescribed protocol (Amersham pharmacia biotech RPN-3000).

RT-PCR (Reverse transcriptase) and nested PCR detection of mRNA-EBV with primer pair EBNA1A. This was done using according to the prescribed protocol Ready-To-Go kit (Amersham pharmacia biotech PCR beads, code No: 27.9555.01).

Additionally, in a parallel study, nasopharyngeal biopsy samples were collected as part of the routine clinical treatment of patients suspected of NPC. All biopsy samples had been taken before treatment and extracted with the informed consent of patients within the diagnostic monitoring study [7].

After collecting, the biopsy samples were cut into two parts. One part was instantly frozen in liquid nitrogen and stored at 80 C°. The other part was recorded in the barraced formalin for diagnostic histopathology.

EBV-positive undifferentiated NPC was confirmed with paraffin-contaminated biopsy material by hybridizing EBER in situ with PNA probes and immuno-histochemical staining LMP1 using mouse monoclonal antibodies OT21C and/or S12.

EBV latent gene PCR

RNAs from biopsy samples were separated using the RNAs protocol according to the manufacturer's instructions (Qiagen) and from cell lines using TRIzol (in vitro gene). RNA integrity was assessed using Agilent Bioanalyzer and was considered intact on the basis of visible lines, corresponding 18S and 28S rRNA.

LMP1, LMP2 and ENBA1 primers and probes TaqMan for PCR were created in real time by Applied Biosystems and included in the sets for Assays-on-Demand gene expression. All primers and probes are listed below. Control reagents TaqMan- β-actin were used as loading control. Reactions of reverse transcription (RT) were conducted using set of reagents for the synthesis of cDNA iScript, and all the samples were processed with DNA-free reagents, according to the manufacturer's instructions. In 20-µl PCR incubated at 95C° for 3 minutes, then 50 cycles at 95C° for 15 seconds and 60C° for 1 minute.

The PCR primers and EBV expression probes were as follows: EBER1 Fwd (5' -ACCGAAGACGGCA GAAAGC-3'), EBER1 Rev (5' -CCTACGCTGCCCTAGAGGTTT-3) and probe EBER1 (5' -6FAMACAGACACCGTCCTCACCACCCG-TAMRA-3'); LMP1 Fwd (5' -ACCACGACACACTGATGAACAC-3'), LMP1 Rev (5' -CTA GAATCGTCGGTAGCTTGTTGA-3') and probe LMP1 (5' -6FAM-ACTCCC TCCCGCACCC-MGB-3'); LMP2 Fwd (5' -TTCTGGCTCTTCTGGGAACAC3'), LMP2 Rev (5' -GGCTCTTCATTAGATTCACGTTCCT-3') and generator LMP2 (5'-6FAM-ACCCCACCGAACGAT-MGB-3') and EBNA1 Fwd (5'-TG AGTCGTCTCCCCTTTGGA-3'), EBNA1 Rev (5'-CCTTAGTGGGCCAGGT TGTG-3') and probe EBNA1 (5'-6FAM-ATGGCCCCTGGACCC-MGB-3').

Multiplex RT-PCR with stem loop

All miRNA EBV sequences were obtained from Sanger's miRNA registry), and the RT stem loop primers were constructed on the basis of these sequences, as described above. The RT stem loop primers for all miRNA EBV were merged so, that the reaction must contain the final concentration of 12.5 nanomolar of each Prime RT.

When one RT primer was used, the final concentration was 50 nanomolar. The set TaqMan MicroRNA RT (Applied Biosystems) was used, and reactions were incubated according to the instructions of the set [8].

The reaction volume of RT was adjusted according to the number of PCR conducted. Final input of RNA biopsy NPC in RT reactions was from 250 to 350 ng.

All RT reactions, including matrix-free control, were held in duplicate. Real-time primers and probes for PCR were created for each miRNA EBV, as described previously. Each PCR with 10µl miRNA included 1 µl RT output, 5µl 2 IQ Supermix (Bio-Rad) [9].

Upstream primer 1,5 µM, universal downstream primer 0,7 µM and probe TaqMan 0,2 µM (Applied Biosystems). Samples incubated in the Bio-Rad cycle MyIQ at 95 ° C for 3 minutes followed by 40 cycles at 95 ° C, within 15 seconds and 60 ° C for 30 seconds.

All real-time PCR were made for the same batch of RT for each sample in duplicate. Taqman miRNA analysis on Rnu6b (Applied Biosystems) was used as boot control [10].

Calculation of miRNA copies per cell

To calculate the number of copies of each miRNA per tumor cell, is needed to know how many cells were used to obtain the input RNA used for the PCR. However, because of their small size, it is not possible to calculate directly number of cells in biopsy samples, therefore, a different approach has been developed for data assessment [11].

First, serial breeding of EBV positive cell line NPC C666-1 and RT-PCR for cell gene expression of gene β-actin and two latent genes EBV EBER1 and LMP1were performed. It was therefore possible to construct the calibration curves C666-1 (number of cells compared to RT-PCR signal) for all three genes. In parallel, RT-PCR was conducted for β-actin gene, EBER1 and LMP1 on the same biopsy samples that were used to measure the number of copies of miRNA EBV [12].

These RT-PCR signals and C666-1 calibration curves used then to calculate cell equivalents C666-1 in each NPC biopsy sample. The analysis of actin makes it possible to estimate the total contribution of cells in PCR miRNA.

However, because NPC tumors have a large neoplastic lymphoid infiltrate, we also used EBER1 and LMP1 analysis to obtain two independent estimates of the total number of EBV-positive, i.e., tumors cells, used for the formation of RNA. Finally, we took the total number of miRNA copies and divided by the estimated number of input cells, derived from the calibration curves C666-1, to estimate the average number of miRNA copies per cell in biopsy samples [13].

Results, and discussion

Profiling miRNA EBV with multiplexed RT-PCR

About 40 mature miRNAs have been described, originated from the BHRF1 and BART regions of the EBV genome. In addition, a new, previously unpublished miRNA (miR-BART22) was revealed using the miRNA cloning protocol [14].

Based on the PCR protocol for detecting miRNA, a sensitive and specific method of multiplexed PCR has been developed to quantify these miRNA EBV in small quantities of material, available from clinical biopsy samples. In this method the primer «stem-loop» for RT is used followed by Taqman real-time PCR using miRNA specific upstream primers, probes and universal downstream primer, specific for the constant region of the prime of the RT stem loop [15].

Using these specifications, original primers and probes for new miRNA EBV BART, and 38 of the 39 miRNA EBV listed in the miRNA database of the Sanger Center at the time, have been successfully developed. In addition, at the result of studies conducted 31 NPC patients were diagnosed by histology in K Hospital and Department of ENT of 103 Hospital in Hanoi, the following results were obtained:

(Table 1)

The patients are mainly of UCNT: 90,31% as some earlier reports of Hanoi K Hospital such as Hoang Xuan Khang (1982): 86,60%.

(Table 2)

- 85,71% are in conformity with UCNT histology.
- 14,29% are not in conformity with UCNT histology.
- 100% are not detected of genome EBV in tissue samples of KSCC and NKC.

Shigeyuki Murono et al (1997) and Li Fu Hu et al (1998): 100% are in conformity with UCNT histology.

Choon-Kook-Sam et al (1996): 76% are in conformity with UCNT histology[17].

Phan Thi Phi Phi et al (1997): 71% are in conformity with UCNT histology.

(Table 3)

96,43% are in conformity with UCNT histology, of which 2/27 cases (7,40%) are positive before histological 6 months and 9 months.

- 3,57% are not in conformity with UCNT histology.
- 100% are not detected EBV genome in tissue samples of KSCC and NKC.

Both PCR and dot blot hybridization: 96,43% are in conformity with UCNT histology.

(Table 4)

- UCNT: 100% (28/28) mRNA – EBV positive.
- NKC: 100% (2/2) mRNA – EBV positive.
- KSCC: 100% (1/1) mRNA – EBV negative.

Shigeyuki Murono et al (1997): Expression of mRNA – EBV was detected by RT – PCR with primer pairs EBNA1A.

UCNT: 100%

NKC: 91,66%

Was not detected in all cases of KSCC.

Halmilton – Dutoit et al (1994): Expression of mRNA – EBV was detected by in situ hybridization with same results comparaing with ours.

Sensitivity and specificity of multiplex analysis

Several tests have been conducted to confirm the usefulness of the analysis and to determine the limits of its sensitivity. Synthetic oligonucleotides representing all miRNA, mixed with a known number of copies and subjected RT.

Each miRNA was analyzed using the fraction extracted from DNA [18].

Except for one PCR, miR-BART14, which was excluded from further analysis, all PCR analyses found miRNA up to 10 copies of synthetic miRNA and showed a linear relationship between number of copies and number of cycles of PCR up to 100 copies.

This is true regardless of whether miRNA was tested separately or in mixtures, containing all 39 miRNA. Furthermore, the sensitivity of the analysis has been not changing, when the RT reaction was carried out using the combined miRNA-specific RT primers (all RT), compared to the use of one miRNAspecific RT (single RT) [19].

We ended up comparing the reproducibility of the detection, when oligonucleotides were analysed separately, or after adding to an entire extract from EBV-negative cells. Again, there were no significant differences (not shown), indicating that all miRNA have been effectively restored and that the low numbers of copies found, for example, for miR-BART2-3p, were not just the result of inefficient recovery of this particular miRNA [20].

The analysis was also very specific. All PCR tested for cross-reactivity using combined RT primers in the pool of all oligonucleotides, including or excluding synthetic oligonucleotide for the tested miRNA [21].

In all PCR, detection of a specific sequence was 106 times more sensitive, than for non-specific / cross hybridized sequences (data not shown). In other words, cross-reacting miRNA would have to be present in the number of copies 108, to generate a cross-signal equivalent to 100 copies of the specific sequences. Direct cloning by many groups revealed single nucleotide end polymorphisms for many miRNA
[22].

It has not yet been determined whether these options are real or artifacts, introduced by cloning. We were concerned that it might affect sensitivity of our analysis, since it was shown, that the efficiency of the PCR in real time “core-loop” can be reduced for variants.

We’ve been researching the effectiveness of PCR in the range of the number of copies for each of

the nine miRNA EBV, the variants of which were listed in miRBase [23].

In all cases, PCR effectively detects variants up to 102 copies. Only in two cases where the new version included deletion, rather than adding one nucleotide, the analysis is less quantitative, and then only with 103 copies. No wonder when the miRNA EBV expression profile was investigated in C666-1 using alternate sequences as standard controls, the profile has not changed significantly (data not shown) [24].

Therefore, this is a potential problem only for miRNA present in the biopsy samples with a low number of copies, such as miR-BART2-3p and miR-BART10, although variants of these miRNA [25] are not currently described.

Conclusion

Having studied 31 biopsies samples of nasopharyngeal carcinoma (NPC), it was found that:

- Histology diagnostic of UCNT is 90,31%.
- Both PCR and dot blot hybridization with primer pair EBNA2A: 1 case of KSCC and 2 cases NKC are negative. However, 96,43% of UCNT cases (27/28) are positive.
- Expression of mRNA – EBV was detected by RT – PCR with primer pairs EBNA1A, it was detected in all 2 cases of NKC and 28 cases of UCNT (100%). Only was not detected in 1 case of KSCC.

In conclusion, EBV and its gene activity are associated to UCNT and NKC but not toKSCC.

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