A New Diagnostic Marker for Secreted Epstein-Barr Virus–Encoded LMP1 and BARF1 Oncoproteins in the Serum and Saliva of Patients with Nasopharyngeal Carcinoma

Karim Houali,[1] Xiaohui Wang,[1] Yuko Shimizu,[1] Djamel Djennaoui,[2] John Nicholls,[3] Sylvie Fiorini,[1] Abdelmadjid Bouguermouh,[4] and Tadamasa Ooka[1]

Abstract

Purpose: EBV has been associated with nasopharyngeal carcinomas (NPC). In North Africa, the incidence is bimodal—the first peak occurring at ~20 years of age and the second peak occurring at ~50 years. Standard diagnostic tests based on immunofluorescence using anti-IgA EBV have shown that young North African patients have a negative serology compared with older patients. We are interested in two EBV-encoded oncoproteins, LMP1 and BARF1, which have thus far not been studied in terms of their potential as diagnostic markers for NPC. These two viral oncoproteins have been detected in cell culture media, so we tested whether they could be detected in the serum and saliva of patients with NPC.

Experimental Design: LMP1 and BARF1 proteins were analyzed in the sera and saliva of young patients and adult patients with NPC from North Africa and China. We then examined whether the secreted proteins had biological activity by analyzing their mitogenic activity.

Results: Both LMP1 and BARF1 were present in the serum and saliva from North African and Chinese patients with NPC. All young North African patients secreted both proteins, whereas 62% and 100% of adult patients secreted LMP1 and BARF1, respectively. From animal studies, the secreted LMP1 was associated with exosome-like vesicles. These secreted EBV oncoproteins showed a powerful mitogenic activity in B cells.

Conclusion: Both proteins will be a good diagnostic marker for NPC whereas BARF1 is a particularly promising marker for all ages of patients with NPC. Their mitogenic activity suggests their implication in the oncogenic development of NPC.

Nasopharyngeal carcinoma (NPC) is a human malignancy derived from the epithelium of the nasopharyngeal cavity. It is one of the most striking examples of a human malignancy that is consistently associated with a virus (1–3). The EBV genome is contained in all malignant NPC cells and it encodes viral proteins that contribute to the malignant phenotype (4–6). Even though infection with EBV is ubiquitous in humans, the incidence of NPC is extremely variable, depending on the geographic area. Whereas the incidence of NPCs in the Chinese population peaks at ~50 years of age, there are two peaks of incidence in North Africa—one at ~20 years of age and the second at ~50 years of age (6). Because of the close association of EBV with NPC, detection of EBV anti-IgA, anti-EA, or anti-VCA by immunofluorescence tests in serum from patients with NPC is used in most Asian countries. However, this test is almost always negative for young North African patients (6). Recent data showed a successful diagnosis of NPC by molecular serology based on EBV-encoded proteins, DNase, thymidine kinase, and p16 VCA used as viral antigens (7–10). Virus load in patient blood has been used as a diagnostic marker for NPC (11, 12), but high levels have been reported in nonneoplastic disorders, gastrointestinal malignancies, and for lymphoproliferative disease (13, 14). We therefore need a more reliable, simpler, and specific diagnostic test for NPC.

Several EBV genes are consistently expressed in NPC biopsies including genes encoding the EBERs, EBNA1, LMP1, LMP2A, BARF0, and BARF1 (4, 15–18). Among them, only LMP1 and BARF1 were capable of inducing malignant transformation in rodent fibroblasts (19, 20), and were thus considered as viral oncogenes. The 21 to 56 amino acid sequence of BARF1 was sufficient to induce malignant transformation and Bcl2 activation (21, 22). LMP1, indispensable for B cell immortalization (23), has been reported in one series to be present in 30% to 50% of NPC biopsies (4). By contrast, a large portion

Authors' Affiliations: [1]Laboratoire de Virologie Moléculaire, UMR5537, Centre National de la Recherche Scientifique, Faculté de Médecine Laennec, Université Lyon-1, Lyon Cedex, France; [2]Service d'ORL, Hopital Mustapha, Algiers, Algeria; [3]Department of Pathology, University of Hong Kong, Pok Fu Lam, Hong Kong; and [4]Institut Pasteur d'Algérie, Sidi-Fredj, Staoueli, Algeria

Received 12/14/06; revised 6/8/07; accepted 6/13/07.

Grant support: Agence National de la Recherche MIME programme, Coopération Inter-universitaire Franco-Algérienne no. 05 MDU 663, and La Ligue contre le cancer (Comité de la Loire). University of Tizi-Ouzou, Algeria (K. Houali), Japan Society for the Promotion of Science (Y. Shimizu), and Fondation Pour la Recherche Médicale (X. Wang).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: K. Houali and X. Wang contributed equally to this work.

Requests for reprints: Tadamasa Ooka, Laboratoire de Virologie Moléculaire, UMR5537, CNRS, Faculté de Médecine Laennec, Université Lyon 1, F-69372, Lyon Cedex 08, France. Phone: 33-47801-1836; Fax: 33-47874-9668; E-mail: ooka@sante.univ-lyon1.fr.

doi:10.1158/1078-0432.CCR-06-2945

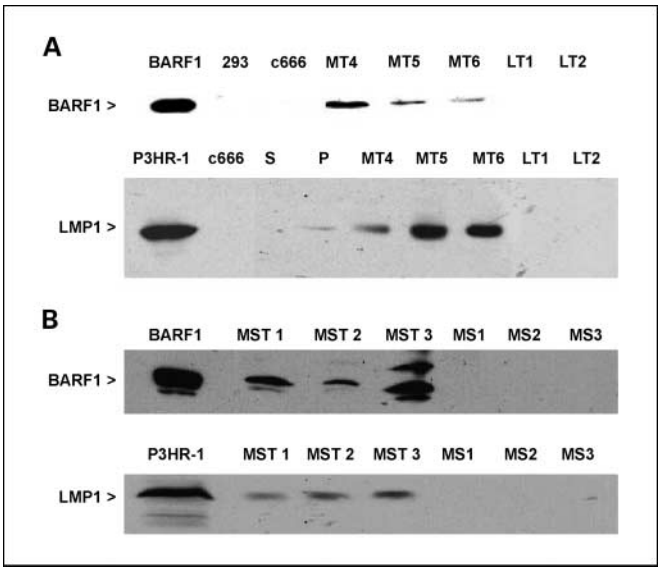

Fig. 1. Detection of BARF1 and LMP1 protein in mice tumor (*A*) and serum (*B*). *A,* BARF1 protein was analyzed in c666-1 cellular extracts (*c666*), in tumors (*MT4-6*), and in cell lines established from tumor biopsy (*LT1-2*). Purified BARF1 protein was used as a control positive (28) and 293 cell extract as a negative control. For LMP1, we prepared exosomal fraction from 12-*O*-tetradecanoylphorbol-13-acetate – treated c666-1 cell culture by sequential centrifugation. C666-1 cells were treated with 20 ng/mL of 12-*O*-tetradecanoylphorbol-13-acetate overnight, then cells were continually cultured for 2 d in serum-free culture medium. S, supernatant from the 110,000 × *g* centrifugation; P, pellet from the 110,000 × *g* centrifugation. We checked culture medium from LT1 and LT2. *B,* three serum samples (*MST1, MST2*, and *MST3*) harvested from nude mice developing the tumors after injection of c666-1 cells. Another three serum samples (*MS1, MS2*, and *MS3*) were from normal nude mice. Approximately 1 mL of serum was harvested from each mouse and 4 μL of each serum was analyzed on 12% SDS-PAGE. BARF1 protein produced from 293 cells – infected recombinant adenovirus was used for BARF1 as positive controls. Antibody anti-BARF1 (Pep-2A: 9) and anti-LMP1 (S12) were used at 1:2,000 and 1:500 dilutions, respectively. Dilution of secondary antibodies was, respectively, 1:5,000 and 1:1,000 for BARF1 antibody. For LMP1 analysis, we prepared exosomes from 200 μL of serum by sequential centrifugation, followed by a sucrose gradient. Semipurified LMP1-exosome was analyzed by immunoblot. For LMP1, a P3HR-1 cell extract was used as a positive control (*P3HR-1*).

(>98%) of NPC biopsies expressed BARF1 by immunohistochemistry and nucleic acid sequence–based amplification (18, 24). Among the viral lytic proteins, BARF1 alone was expressed consistently and at high levels in NPC- and EBV-associated gastric carcinomas as well as in EBV-immortalized epithelial cells *in vitro* (25–27). On the other hand, BARF1 was able to immortalize primary monkey kidney epithelial cells *in vitro* (28). Therefore, BARF1 likely plays an important role in epithelial oncogenesis. BARF1 protein, a hexamer oligomeric structure as determined by crystallography study (29), acts as a powerful mitogen (30). The BARF1 protein can complex *in vitro* with colony-stimulating factor 1, resulting in the inhibition of macrophage activation (31), and can also inhibit the secretion of IFN-α in EBV-infected B cells (32). BARF1 is therefore involved not only in oncogenic development, but also in immunomodulation.

The other postulated EBV oncogene, LMP1, is essential for B cell immortalization, activating several cellular genes such as nuclear factor κB, A20, and epidermal growth factor receptor (33–35). It can inhibit cell differentiation when transfected into epithelial cells (36). Recent data has shown that LMP1 could be secreted and was localized in the exosomal component in culture medium of B95-8 cells, as well as in insect Sf9 cells infected with LMP1 recombinant Baculovirus (37, 38). These exosomal components were likely responsible for the inhibition of T cell proliferation (39).

As BARF1 and LMP1-exosomes are secreted in the culture medium, we asked whether they are secreted in the serum as well as in the saliva of patients with NPC. Our hypothesis is that the secreted proteins in serum can play a crucial role for cell activation, immunomodulation, and/or immunosuppression. We report here the secretion of both these oncoproteins in the serum and saliva of patients with NPC. These proteins (BARF1 protein and LMP1 complexed with exosome) showed powerful mitogenic activity *in vitro*.

Materials and Methods

Cell culture. NPC tumor–derived c666-1 (40) and P3HR-1 cells were cultured in RPMI 1640 with 10% FCS, streptomycin, and penicillin.

Serums and saliva. Sera (n = 250) and saliva (n = 50) from Algerian patients with NPC at different ages as well as sera from healthy individuals (n = 50) were collected in Mustapha Hospital (Algiers, Algeria). Sera from healthy French individuals (n = 100) were also purchased from Centre de Transfusion Sanguine in Lyon. Sera from Chinese patients with NPC (n = 30) were obtained from The University of Hong Kong courtesy of Professor M.H. Ng (Department of Pathology, University of Hong Kong, China).

Tumor induction in nude mice. As previously described (20), freshly harvested c666-1 cells were washed with serum-free RPMI 1640. Cells ($10\text{-}20 \times 10^6$) were injected s.c. into nude mice. After 3 weeks, a ~2 cm diameter of tumor biopsy was harvested. Approximately 1 mL of blood was also harvested per mouse and centrifuged at 3,000 rpm for 20 min followed by centrifugation at 10,000 rpm for 1 h to obtain clear serum.

Extraction of protein from mice. Tumor fragments were cut into small pieces and put in a radioimmunoprecipitation assay buffer with antiprotease (20). The samples were sonicated twice for 15 s (20 W amplitude), then clarified by centrifugation at 10,000 rpm for 5 min.

Sucrose gradient. Before loading onto a sucrose gradient, serum samples were treated with protein A to eliminate a large part of the immunoglobulins (25 and 50 kDa) that compete for the detection of viral proteins, LMP1 and BARF1, which have a similar molecular weight. As previously described (30), 100 μL of protein A–treated mice serum or 1 mL of protein A–treated NPC serum was loaded onto 5 mL of a 5% to 40% linear sucrose gradient. After gradient ultracentrifugation at 105,000 × *g* for 16 h, 100 μL (for 5 mL of gradient) and 200 μL (for 10 mL of gradient) for each fraction were collected. LMP1 and BARF1 proteins were localized by immunoblot.

Preparation of exosome. One milliliter of NPC serum or 100 μL of mouse serum as well as 500-fold concentrated c666-1 culture medium were treated five times with 1 μg of protein A, then supernatant was centrifuged at 12,000 × *g* for 45 min. The supernatant was recentrifuged at 70,000 × *g* for 1 h. Lastly, the supernatant was centrifuged at 110,000 × *g* for 2 h. The pellet was dissolved in PBS, then filtered with a 0.22-μm filter. The filtered solution was centrifuged again at 110,000 × *g* for 2 h. The pellet was dissolved in PBS and loaded at the bottom of 5% to 40% of a 10 mL sucrose gradient (41, 42). After centrifugation at 100,000 × *g* for 15 h, 50 fractions were collected. Each fraction was analyzed by anti-LMP1 antibody. LMP1 was detected in the 35th to 45th fraction. LMP1-positive fractions were combined and centrifuged at 110,000 × *g* for 1 h. The pellet was placed at the bottom of the centrifugation tube, then a sucrose gradient of 5% to 40% (10 mL) was loaded over it and the samples centrifuged at 105,000 × *g* for 15 h at 4°C, and 200 μL fractions were collected. The fractions containing exosome were combined and used for immunoblot analysis or immunoelectron microscopy.

Immunoelectron microscopy. The pellet was washed twice with PBS, then resuspended in 20 μL of 0.02% sodium azide/PBS, fixed with 2% paraformaldehyde, placed on copper grids, and air-dried. Grids were

blocked with bovine serum albumin/PBS for 30 min, incubated in antimouse anti-LMP1 S12 antibody, or in rabbit polyclonal anti-LMP1 antibody (a gift from Dr. J. Middeldorp, Department of Pathology, Free University of Amsterdam, Amsterdam, Holland) for 45 min and labeled with secondary antibody (antirabbit antibody conjugated with 10 or 20 nm gold grains) for 45 min. Grids were stained with 1% uranyl acetate and visualized with electron microscopy type JEOL 1200 CX operating at 80 kV.

Immunoblotting. As previously described (19), 50 or 70 μg of proteins were separated in 10% or 12% polyacrylamide gels and blotted onto nitrocellulose. Nonspecific protein-binding sites were blocked by overnight incubation of blotted filters in TBS buffer with 5% lyophilized bovine serum albumin (Sigma). The filters were subsequently incubated overnight at 4°C with anti–Pep-2B for anti-BARF1 (19) and antirabbit OT20T or S12 for LMP1. The filters were then washed and incubated for 1 to 2 h at room temperature with peroxidase-labeled polyclonal antirabbit IgG. The antigen-antibody complexes were then visualized using an enhanced chemiluminescence system (Amersham) according to the instructions of the manufacturer.

Extraction of LMP1 from BJAB and Raji cells. LMP1 was extracted from 2×10^7 Raji cells by agitation for 15 min at 4°C in 1 mL of a buffer containing 0.05 mol/L of sodium acetate (pH 6.0), 0.22 mol/L of octyl-β-glucoside (Pierce), and 3% urea. The mixture was sonicated twice for 15 s (20 W amplitude), then clarified by centrifugation at 13,000 rpm for 5 min. The supernatant was used for ELISA assay. For negative controls, the same fraction was prepared from EBV-negative BJAB cells.

ELISA test. Two hundred microliters of polyclonal OT20T LMP1 antibody (diluted to 1:1,000) or anti–Pep-2B antibody raised against the NGGVMKEKD peptide (amino acids 172-180) of BARF1 protein (19) were coated onto a 96-well microplate overnight at 4°C. Plates were washed four times with PBS containing 0.05% Tween and 200 mL of 1% bovine serum albumin was added. After 2 to 4 h incubation at 4°C, bovine serum albumin was eliminated by washing four times with PBS-Tween 20 at 0.05%. One hundred microliters of human serum from patients with NPC or from healthy individuals was added and incubated for 30 min at 37°C with agitation. Antibody was eliminated by washing four times with PBS containing Tween 20. One hundred microliters of monoclonal anti-LMP1 S12 or monoclonal anti-BARF1 were added, then incubated for 30 min at 37°C with agitation. Antibody was then eliminated by washing four times with PBS-Tween 20. One hundred microliters of peroxidase-conjugated antimouse antibody were added, then incubated for 30 min at 37°C with agitation. The wells were washed with PBS-Tween 20, then o-phenylenediamine dihydrochloride (ODP) was added and incubated for 30 min at 37°C with agitation. The reaction was stopped by the addition of 25 μL of 5 mol/L of sulfuric acid, and absorbance was read at 490 nm by an ELISA reading apparatus. BJAB (EBV-negative cells) and Raji (EBV-positive cells) were used for LMP1 as negative and positive controls. Purified p29 BARF1 protein was used as a positive control.

Mitogenic activity analysis. As previously described (29), cell proliferation and viability were tested using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (cell proliferation kit; Roche). Human Louckes B cells were seeded at 10^4 cells/well in 96-well culture plates (BD Labware), and cultured in 100 μL of RPMI 1640 with 10% FCS. The culture medium was then discarded, and the cells were washed four times with PBS. Each well then received 10 μL of mouse serum containing BARF1 protein or LMP1 in 100 μL of serum-free RPMI 1640. At 48 h of culture, 10 μL of the MTT solution was added per well. After 4 h of incubation, colored crystals of formazan were dissolved overnight at 37°C with a 100 μL solubilization solution, and absorbance was measured at 600 nm. The same experiment was carried out on NPC serum and saliva. For NPC serum, BARF1 from the sucrose gradient fraction was further purified with a concanavalin A column, and we also prepared LMP1-exosome by using a sucrose gradient starting from 5 mL of NPC serum. To examine if mitogenic activity observed with LMP1-exosome and BARF1 was due to LMP1 or BARF1, S12 anti-LMP1 or anti-BARF1 was added to the reaction.

Results

Secretion of BARF1 protein and LMP1 in serum and tumors of nude mice after injection of EBV-positive c666-1 NPC epithelial cells and tumor formation. To investigate the secretion of BARF1 and LMP1 in serum, we used the NPC-derived EBV-positive c666-1 epithelial cell line (40). This epithelial cell line

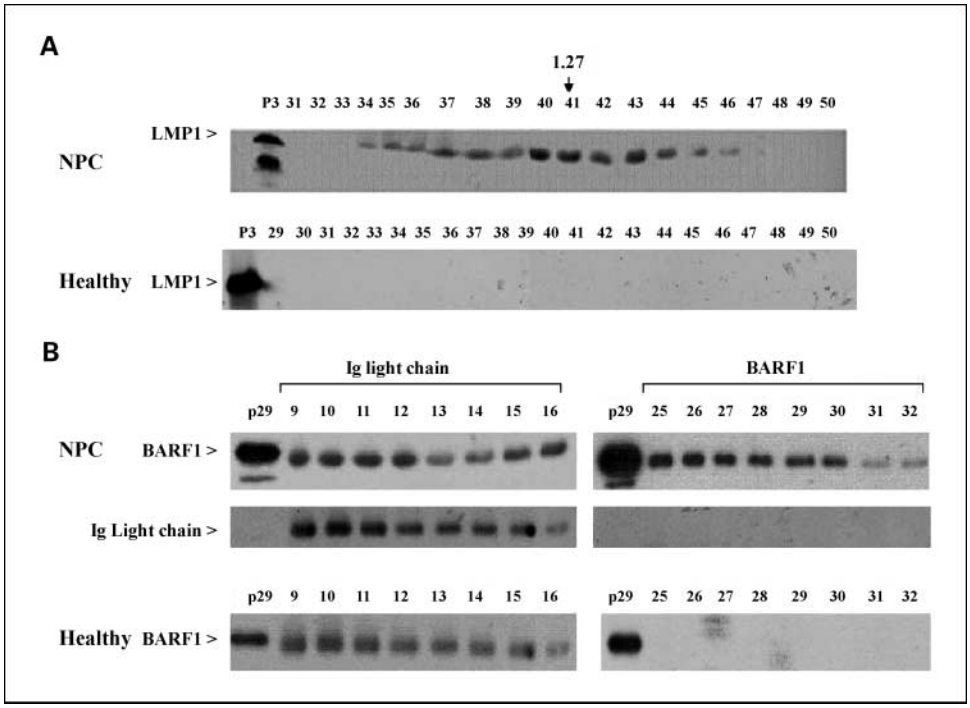

Fig. 2. LMP1 and BARF1 localization in sucrose gradient. One hundred microliters of NPC serum was loaded onto 5 mL of a 5% to 40% linear sucrose gradient. After gradient ultracentrifugation at 105,000 × *g* for 16 h, 100 μL of each fraction was collected. Each fraction was analyzed on immunoblot using S12 antibody (*A*) or Pep-2A (*B*) for LMP1 or BARF1, respectively. LMP1-exosome was found mainly at a density of 1.27 g/mL. BARF1 antibody revealed positivity for the 9th to 16th fraction (*B, NPC, BARF1*) which also contains immunoglobulin. The same positive response was also found in the same fractions with serum from healthy donors (*B, Healthy*). With anti-BARF1, fractions 25 to 32 became positive with NPC serum, whereas such positive bands were never found with serum from healthy donor (*B, Healthy, BARF1, bottom*). Positive responses on fractions 9 to 16 with BARF1 antibody were due to immunoglobulin light chain recognized by anti-BARF1 antibody as anti-IgG could recognize fractions 9 to 16, but not fractions 25 to 32 (*B, IgG light chain*).

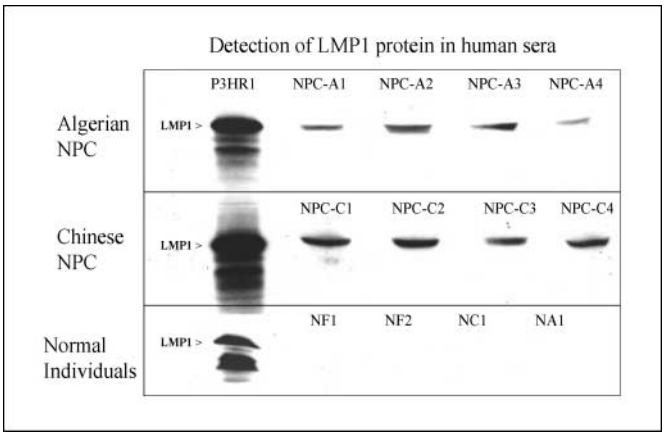

Fig. 3. Presence of LMP1 in NPC serum from Algerian and Chinese patients. Sixty microliters of serum from patients with NPC were treated five times with 200 μL of 10% protein A. Five microliters of each protein A – treated serum was analyzed on 12% SDS-PAGE, then by immunoblot. LMP1 was revealed with anti-LMP1 S12 antibody. NPC-A, Algerian patient with NPC; NPC-C, Chinese patient with NPC; NF1, EBV-positive healthy French individual; NF2, EBV-negative healthy French individual; NC, healthy Chinese individual; NA, healthy Algerian individual.

harboring the EBV genome developed a tumor when injected into nude mice. Using this animal model, LMP1 and BARF1 expression was analyzed in the c666-1 cell line (*in vitro*), in induced tumors (*in vivo*), and in a tumor cell line established from mouse tumors (*in vitro*). When LMP1 and BARF1 expressions were compared in these materials, BARF1 and LMP1 expression was negative in the c666-1 cell extract (Fig. 1A, *c666*), whereas their expression became positive during tumor development (*MT4*, *MT5*, *MT6*; Fig. 1A, *top*, *BARF1*). Their expression became almost undetectable in cellular extracts from the established tumor cell lines (Fig. 1A, *LT1* and *LT2*). However, the absence of BARF1 protein in c666-1 cells was not due to the absence of its transcription as we could detect its transcription by reverse transcription-PCR (data not shown). Our findings suggest that BARF1 protein was synthesized in c666-1 cells, then secreted into the culture medium. LMP1 was also difficult to visualize in cellular extracts from c666-1 and c666-1 tumor cell lines LT1/LT2 (Fig. 1A, *bottom*). However, LMP1 became detectable when we prepared exosomes from the culture medium of c666-1 cells (Fig. 1A, *P*) indicating that LMP1 associated with exosome-like vesicles were secreted from epithelial c666-1 cells. LMP1 also became detectable in the culture medium of tumor cell lines (*LT1/LT2*) when we prepared exosomes from the culture medium (data not shown). LMP1 and BARF1 expression is quite different *in vitro* and *in vivo* depending on the epithelial environment.

If BARF1 and LMP1 are secreted *in vitro*, we therefore asked whether LMP1 and BARF1 are secreted into the mouse serum. As LMP1 was detected in association with exosome in the c666-1 culture medium, we examined its presence in serum from mice who had developed tumors. The presence of both proteins was analyzed by immunoblotting. All sera from mice developing tumors contained 29 kDa of BARF1 protein (Fig. 1B, *MST 1-3*, *top*), whereas such proteins were never found in normal mice (*MS 1-3*). To visualize the 29 kDa BARF1 protein on immunoblot, we treated serum several times with protein A to eliminate the 25-kDa light chain immunoglobulins which masked the BARF1 protein. Secreted native BARF1 in serum showed high a molecular weight of ~180 to 240 kDa using sucrose gradient (5-40%) in non-denatured condition (30), with adenoviral BARF1 recombinant indicating its hexameric form (28). For LMP1 detection, it was also very difficult to visualize on immunoblots using bulk serum because of the massive presence of albumin and immunoglobulins. For this reason, we purified exosomes from each serum by differential centrifugation following a 5% to 40% sucrose gradient (see Materials and Methods) to avoid any possible contamination by albumin and immunoglobulins. The exosomes were found at a density between 1.25 and 1.27 g/mL, as expected (42), and LMP1 was detected in the same fractions. Exosome-associated LMP1 was found only in serum from mice developing tumors (Fig. 1B, *bottom*, *MST 1-3*). Secreted LMP1 and BARF1 proteins found in the serum did not come from apoptotic c666-1 cells because we could not find any EBV genome in mice serum by PCR analysis, nor could we find the EBNA1 protein (data not shown).

Secretion of BARF1 protein and LMP1 in serum and saliva of young and adult patients with NPC. As BARF1 and LMP1 were found in the serum from NPC in mice, we asked whether both

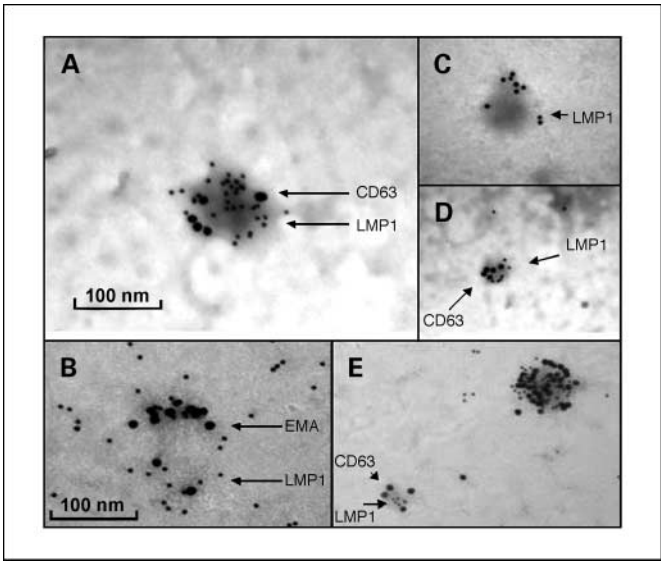

Fig. 4. Detection of LMP1 in exosome-like vesicule by immunoelectron microscopy. One milliliter of NPC serum or 100 μL of serum from mice developing tumors as well as 500-fold concentrated c666-1 culture medium were treated with 42 μg of protein A five times, then supernatant was loaded onto 5% to 40% of sucrose gradient. After centrifugation at 100,000 × *g* for 15 h, 50 fractions were collected. Each fraction was analyzed by anti-LMP1 antibody. LMP1 was detected in the 37th to 45th fractions (see Fig. 2). LMP1-positive fractions were assembled and centrifuged at 110,000 × *g* for 1 h. The pellet was washed twice with PBS, then resuspended in 20 μL of 0.02% sodium azide/PBS, fixed with 2% paraformaldehyde, placed on copper grids, and air-dried. Grids were blocked with 1% bovine serum albumin/PBS for 30 min, incubated with rabbit polyclonal anti-LMP1 antibody for 45 min, and labeled with secondary antibody (anti-rabbit antibody conjugated with 10 nm of gold bead for LMP1 and with 20 nm of gold bead for CD63 or EMA) for 45 min. Grids were stained with 1% uranyl acetate and visualized with electron microscopy (Siemens) operating at 80 kV. Positive grains were seen in NPC serum, pellets from c666-1 culture medium, and serum from mice developing NPC-derived tumors. LMP1 was localized in exosome-like vesicules with a diameter varying between 60 and 100 nm (*black arrow*). *A,* from NPC serum with double-labeling with LMP1 (10 nm gold bead) and CD63 (20 nm gold bead). *B,* NPC serum with double-labeling with LMP1 (10 nm gold bead) and EMA (20 nm gold bead). *C,* NPC serum. *D,* c666-1 culture medium with double-labeling (10 nm gold bead) and CD63 (20 nm gold bead). *E,* mouse serum developing NPC-derived tumor with double-labeling (10 nm gold bead) and CD63 (20 nm gold bead).

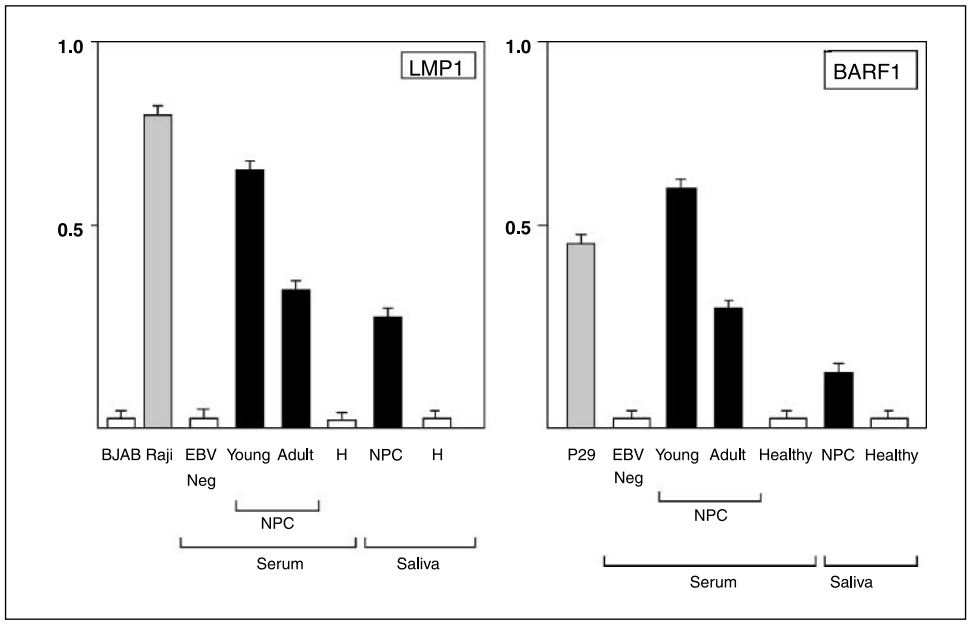

Fig. 5. Detection of LMP1 and BARF1 protein by ELISA test on NPC serum and saliva. For LMP1, monoclonal LMP1 antibody was coated, then NPC serum or saliva was added. After washing, polyclonal anti-LMP1 was added. Membrane extract from Raji cells was used as a positive control. BJAB membrane extract and EBV-negative serum as negative controls. One hundred microliters of serum and 100 μL of saliva (diluted by PBS after centrifugation at 10,000 × *g* for 20 min) were analyzed. For the saliva test, 30 samples of saliva from patients with NPC and 25 samples of saliva from healthy Algerian and French individuals were used. *Y*-axis, the value of absorbance at 490 nm. The *P* values were 0.023, 0.0134, 0.0342, 0.0262, 0.0750, 0.0573, 0.0297, and 0.0323 for BJAB, Raji, EBV-negative serum, young patient with NPC, adult patient with NPC, healthy individuals, saliva of patients with NPC, and saliva of healthy individuals, respectively. For BARF1, polyclonal anti-BARF1 (pep-2B antibody; ref. 17) was coated, then NPC serum or saliva was added. After washing, monoclonal anti-BARF1 was added. P29 BARF1 protein purified from culture medium of 293 cells infected by BARF1 recombinant adenovirus was used as a positive control and EBV-negative healthy serum as a negative control. The *P* values were 0.0259, 0.0531, 0.0654, 0.0234, 0.0154, 0.0278 and 0.0871 for p29, EBV-negative serum, young patient with NPC, adult patient with NPC, healthy individuals, saliva of patient with NPC, and saliva of healthy individuals, respectively. The value was presented as an average of 250 and 300 sera from young and adult patients with NPC, respectively.

proteins were also secreted in the serum of patients with NPC. We examined their presence in bulk serum from young and adult Algerian patients with NPC on immunoblot. To yet again avoid masking of detection by immunoglobulins and albumin, we semipurified BARF1 and LMP1-exosomes by sucrose gradient (Fig. 2).

For BARF1, 1 mL of NPC serum (treated several times with protein A) was loaded onto a 5% to 40% sucrose gradient. Fractions from the 25th to the 30th sucrose gradient (Fig. 2B) were pooled, concentrated thrice, and examined by immunoblot. We could detect the 29 kDa BARF1 protein. This band did not come from contaminated immunoglobulins because when we incubated the blot with antihuman immunoglobulin antibody, this band did not show up (immunoglobulin light chain, BARF1). Moreover, sera harvested from healthy donors did not produce any band in the same gradient fractions (25-32th gradient). BARF1 protein was therefore secreted in the serum of patients with NPC.

For LMP1, exosomes semipurified by differential centrifugation from Chinese and North African patients with NPC were loaded onto 5% to 40% sucrose gradient. After centrifugation, LMP1 was found in similar fractions as those of mouse serum. After centrifugation at 110,000 × *g*, the pellet containing LMP1-exosome was first analyzed by immunoblotting (Fig. 2A). Immunoblot analysis showed that only serum from Algerian patients with NPC gave a positive response (the main band was detected at a density of 1.27 g/mL, whereas no such bands were revealed with serum from healthy individuals; Fig. 2A, *Healthy*). When we compared Algerian, Chinese, and healthy donors, we could clearly detect LMP1 in patients with NPC, whereas sera from French (*NF*), Chinese (*NC*), and Algerian (*NA*) healthy donors did not contain any LMP1 (Fig. 3).

Immunoelectron microscopy analysis revealed that LMP1 protein was localized in vesicle-like components ~ 50 to 100 nm in diameter (Fig. 4A, B, C, NPC sera). Similar experiments were carried out on sera harvested from tumors developing in mice (Fig. 4D) as well as on LMP1-exosomes secreted in 12-*O*-tetradecanoylphorbol-13-acetate – treated c666-1 cell culture mediums (Fig. 4E). To confirm that LMP1 was associated with exosome-like vesicles, we analyzed exosomes by double-labeling them with CD63 (an exosome marker) and LMP1. We also analyzed whether exosome-like components were of epithelial origin in examining the presence of epithelial membrane antigen (EMA) on exosome-like components. The simultaneous presence of LMP1/CD63 (Fig. 4A) or LMP1/EMA (Fig. 4B) was visualized by double immunogold labeling. More than 40% of exosomes were found to be positive for LMP1/EMA/CD63, indicating that they were likely of epithelial origin.

We then investigated whether both viral proteins were also unique in the saliva of patients with NPC. Immunoblotting revealed that BARF1 and LMP1 were found only in patients with NPC, whereas saliva from healthy donors did not contain a significant amount of LMP1 or BARF1 proteins.

Quantitative analysis of BARF1 protein and LMP1 by ELISA test. To confirm the presence of LMP1 and BARF1 proteins in serum and saliva, we analyzed their origins by examining the presence of EMA on exosome-like by ELISA method. For BARF1 detection, using anti – Pep-IIIB BARF1 antibody and monoclonal BARF1 antibody, sandwich ELISA was carried out on sera

and saliva. We used purified p29 BARF1 protein as a positive control.

All sera derived from patients with NPC from Algeria and Hong Kong contained high levels of BARF1 protein, whereas sera from healthy donors gave background values similar to those of sera from EBV-negative individuals (Fig. 5, BARF1). The level of BARF1 was much higher for young patients than adult patients.

LMP1 ELISA gave similar results as those of BARF1 (Fig. 5, *LMP1*). Approximately 38% of all adult patients with NPC tested in our study did not show any positive response, whereas all sera from young patients with NPC were positive. LMP1 protein levels were also much higher in young patients than in adult patients as observed with BARF1. From ELISA tests, the quantity of LMP1 and BARF1 proteins in NPC sera was ~2 and 5 μg/mL of serum, respectively, for LMP1 and BARF1 protein from young patients, and 0.2 and 0.5 μg, respectively, for LMP1 and BARF1 from adult patients.

Analysis of saliva from 50 patients with NPC showed a significant quantity of both viral oncoproteins. As in the case of serum, the saliva from young patients contained much higher levels of both proteins compared with adult patients. Saliva from 15 Algerian healthy donors were negative for both proteins.

Mitogenic activity of secreted BARF1 protein and LMP1-exosome isolated from mice and NPC serum. Our previous data showed that purified BARF1 protein had mitogenic activity (30). To examine whether BARF1 found in NPC serum was biologically active, human Louckes B cells were incubated in serum-free medium with either 10% FCS, mouse serum containing BARF1, or serum from normal mice. As shown in Fig. 6, cell mitogenic activity measured by MTT assays (30) was efficiently stimulated in the presence of 10% mouse serum (with tumor), whereas serum from normal mice did not show any cell stimulation (Fig. 6A). To check if this effect was due to the BARF1 product, the test was repeated by adding an affinity-purified fraction of our rabbit antibody (18). Seventy percent of the mitogenic activity from the MTT value was inhibited by BARF1 antibody. When we combined the BARF1 antibody with anti-LMP1, total activity was abolished. This suggested that LMP1 and BARF1 could intervene in mitogenic activity. We prepared purified BARF1 and LMP1-exosome from the serum of patients with NPC and tested their mitogenic activity (Fig. 6). Both proteins were prepared from sucrose gradient and concanavalin A affinity column. As illustrated in Fig. 6B and C, semipurified BARF1 (B) and LMP1-exosome (C) were capable of activating the cell cycle. The addition of anti-BARF1 or anti-LMP1 abolished this mitogenic activity. The same gradient fractions from the serum of healthy individuals did not show any mitogenic activity. These data suggested that BARF1 present in the serum of patients with NPC was similar to BARF1 expressed *in vitro* by BARF1 recombinant adenovirus (30). Thus, we have shown for the first time that mitogenic activity associated with LMP1-exosomes could be extracted from NPC serum.

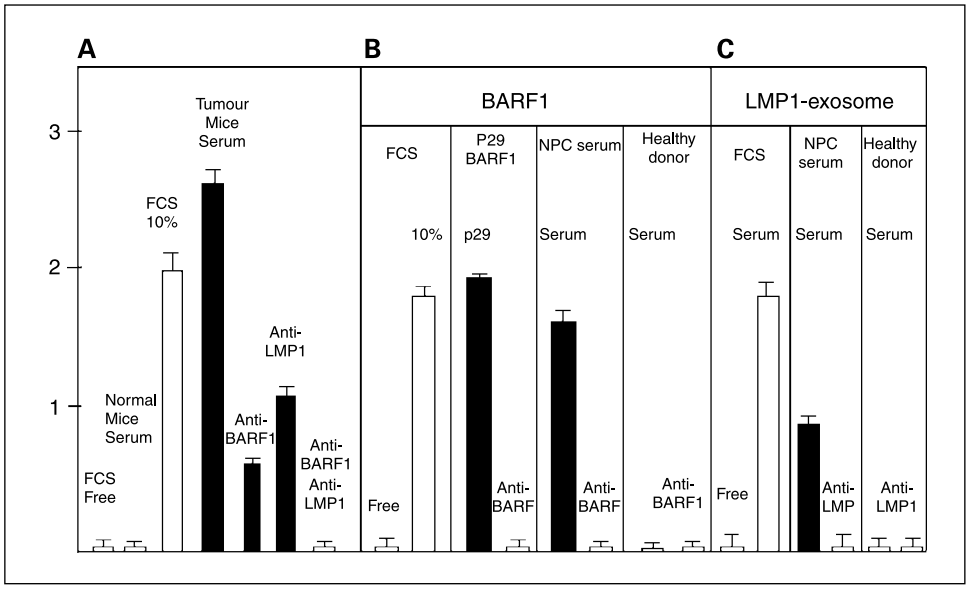

Fig. 6. Cell cycle activation assays by MTT test for mice serum and NPC serum. *A,* human Louckes B cells and rodent BALB/c3T3 fibroblasts were cultured in a 96-well plate (10^4 cells/100 μL complete medium) until 60% to 70% confluence, carefully washed with PBS, and incubated in 100 μL serum-free medium containing 10 μL of mice serum (from tumor-bearing mice or normal mice). As a positive control 10% FCS was used (*FCS 10%*). Negative controls consisted of cells cultured with serum-free DMEM only (0% FCS). To examine the effect of antibodies against BARF1 on cell cycle activation, 2 μL of affinity-purified anti-BARF1 antibody (Pep-2A; ref. 17) were added at the same time with mice serum. Two milliliters of anti-LMP1 S12 antibody were added. After 48 h of incubation, MTT assay was done (29). The *P* values were 0.0321, 0.0754, 0.0621, 0.0579, 0.0254, 0.0324, and 0.0145 for FCS-free, normal mice serum, 10% FCS, tumor mice serum, anti-BARF1, anti-LMP1, and anti-BARF1 + anti-LMP1, respectively. *B,* BARF1 was semipurified by protein A treatment, followed by sucrose gradient (see fractions 25 to 30; Fig. 2). FCS at 10% and p29 BARF1 protein were used as controls. Five milliliters of anti-BARF1 antibody was added to inactivate BARF1. After 48 h of incubation, MTT assay was done as previously described. The *P* values were 0.123, 0.0234, 0.0142, 0.0268, 0.035, 0.0273, 0.0197, and 0.0221 for FCS-free, 10% FCS, p29, p29 + anti-BARF1, NPC serum, NPC serum + anti-BARF1, healthy donor, and healthy donor + anti-BARF1, respectively. *C,* LMP1-exosome was also prepared by protein A treatment, differential centrifugation, and sucrose gradient (see Fig. 2). Ten milliliters (4 μg protein) of LMP1-exosome fraction was added to the reaction. Two milliliters of anti-LMP1 S12 antibody was added to inactivate LMP1. After 48 h of incubation, MTT assay was as previously described. The *P* values were 0.249, 0.0334, 0.0290, 0.0541, 0.0217, and 0.0231 for FCS-free, 10% FCS, NPC serum, NPC serum + anti-LMP1, healthy donor, and healthy donor + anti-LMP1, respectively.

Discussion

The work presented here provides evidence of EBV gene expression in NPC tumors induced in nude mice by the injection of c666-1 cells. This animal model permitted us to show the differences in the expression of two EBV oncogenes (LMP1 and BARF1) *in vitro* and *in vivo*. The expression of these oncogenes was significantly activated during tumor development, indicating the importance of their role during NPC development. We recently reported specific activation of several EBV early genes during tumor development in nude mice after the injection of EBV-positive AKATA B cells (22). This suggests that viral gene expression was controlled by different mechanisms *in vitro* and *in vivo*. The mouse model permitted us to show the secretion of two EBV oncoproteins in the serum of patients with NPC.

LMP1 found in serum from both mice and humans with NPC was associated with exosome-like vesicles that were positive for CD63. All LMP1 proteins were principally found in the exosomal fraction and were principally derived from the NPC because (*a*) LMP1-carrying exosomes in the NPC serum were also positive for EMA, and (*b*) we could find LMP1-positive exosome-like vesicles in serum from nude mice which developed NPC-derived c666-1 tumors. A minor component of the vesicles harboring LMP1 found in NPC serum are of EBV-positive B cell origin, as the presence of B cell–derived exosomes in the serum was recently reported (41). However, there have been no reports thus far about exosomes containing viral proteins secreted in human serum.

Our previous investigations showed that it was very difficult to diagnose young North African patients with NPC by classic immunofluorescence method using anti-IgA/IgG, anti-EA, or anti-VCA (6, 9). In fact, these antibodies showed almost no reaction with the young patients' sera. In contrast, we showed higher levels of LMP1 and BARF1 proteins in serum from young patients with NPC than from adult patients. The detection of LMP1 and BARF1 proteins in serum could therefore be used as an alternative diagnostic test for NPC, in particular, for young patients. Because BARF1 is present in young patients as well as in old patients with NPC, the detection of BARF1 protein in the serum would be a powerful diagnostic marker for all NPCs.

LMP1-positive exosomes were capable of activating the cell cycle in human Louckes B cells. A similar mitogenic activity was also observed with BALB/c3T3 rodent fibroblasts. This activation was due to LMP1 because mitogenic activity was almost totally inhibited by the addition of anti-LMP1 antibody. We also tried to determine whether free LMP1 (without association with exosomes) was capable of activating the cell cycle. However, free LMP1 was not able to activate Louckes cells. Recent data showed that LMP1-exosomes purified from the baculoviral system could suppress primary T cell function (39). On the other hand, exosomes isolated from B cells could activate T cells (42). This suggest that the presence of exosomes is necessary for the mitogenic or suppressive activity of LMP1. Further study will be necessary to understand the exact role of LMP1 in association with exosomes.

We found the secretion of BARF1 and LMP1 proteins in mice serum and from tumor biopsies. These oncoproteins will likely be necessary for the tumor microenvironment.

Remarkably, BARF1 present in mice serum was a functional protein which was able to activate the cell cycle. Recent data showed that BARF1 could neutralize the function of colony-stimulating factor 1 in macrophage activation (31). In this regard, the secretion of BARF1 in serum could have two significant functions: (*a*) to neutralize colony-stimulating factor 1 function to protect virus-infected cells and/or (*b*) to activate the cell cycle by a paracrine mechanism. Activation of the cell cycle by BARF1 and/or LMP1-exosome would be an important step for NPC carcinogenesis. Injection of anti-BARF1 and/or anti-LMP1 antibody in nude mice should thus be tested to determine if the tumor development is inhibited. The mechanism of mitogenic activity by LMP1-exosomes will be further studied by proteomic analysis of the complex (43).

Acknowledgments

We thank Dr. J. Middeldorp (Free University of Amsterdam) for anti-LMP1 antibody and Dr. Pierre Boulanger (UMR5537, CNRS, Lyon) for critical reading.

The study sponsors had no role in the experimental design or the collection, analysis, and interpretation of the data or in writing or submitting the manuscript.

References

1. Zur Hausen H, Schulte-Holthausen H, Klein G, et al. EBV DNA in biopsies of Burkitt tumours and anaplastic carcinoma of the nasopharynx. Nature 1970;228:1056–8.
2. Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. Nat Rev Cancer 2004;4:757–68.
3. Bouzid M, Dennaoui D, Dubreuil C, et al. Epstein-Barr virus genotypes in NPC biopsies from North Africa. Int J Cancer 1994;56:1–6.
4. Fahraeus R, Fu HL, Ernberg I, et al. Expression of Epstein-Barr virus-encoded proteins in nasopharyngeal carcinoma. Int J Cancer 1988;42:329–8.
5. Young LS, Dawson CW, Clar D, et al. Epstein-Barr virus gene expression in nasopharyngeal carcinoma. J Gen Virol 1988;69:1051–65.
6. Sbih-Lammali F, Bouguermouh M, Decaussin G, Ooka T. Transcriptional expression of Epstein-Barr virus genes and proto-oncogenes in Algerian nasopharyngeal carcinoma. J Med Virol 1996;49:7–14.
7. Stolzenberg MC, Debbouze S, NG M, et al. Purified recombinant EBV deoxyribonuclease in serological diagnosis of nasopharyngeal carcinoma. Int J Cancer 1996;66:337–41.
8. De Turenne-Tessier M, Ooka T, Calender A, De Thé G, Daillie J. Relationship between nasopharyngeal carcinoma and high antibody titers to Epstein-Barr virus-specific thymidine kinase. Int J Cancer 1989;43:45–8.
9. Ooka T, De Turenne-Tessier M, Stolzenberg MC. Relationship between antibody production to Epstein-Barr virus (EBV) early antigens and various EBV-related diseases. In: Ooka T, Sixbey JW, editors. Springer Semin Immunopathol 1991;13:233–47.
10. Karray H, Ayadi W, Fki L, et al. Comparison of three different serological techniques for primary diagnosis and monitoring of nasopharyngeal carcinoma in two age groups from Tunisia. J Med Virol 2005;75:593–602.
11. Lo YM, Chan LY, Lo KW, et al. Quantitative analysis of cell-free Epstein-Barr virus DNA in plasma of patients with nasopharyngeal carcinoma. Cancer Res 1999;59:5452–5.
12. Xie Y, Huang G, Zhang Z, Wen W, Wu Y, Wei Z. Application of EB virus latent membrane protein 1 in nasopharyngeal swab in diagnosis of nasopharyngeal carcinoma. J Clin Otorhinolaryngol 2006;20:499–501.
13. Lei KI, Chan LY, Chan WY, Johnson PJ, Lo YM. Quantitative analysis of circulating cell-free Epstein-Barr virus (EBV) DNA levels in patients with EBV-associated lymphoid malignancies. Br J Haematol 2000;111:239–46.
14. Stevens SJ, Verschuuren EA, Verkuujlen SA, Van Den Brule AJ, Meijer CJ, Middeldorp JM. Role of Epstein-Barr virus DNA load monitoring in prevention and early detection of post-transplant lymphoproliferative disease. Leuk Lymphoma 2002;43:831–40.
15. Busson P, McCoy R, Sadler R, Gilligan K, Tursz T, Raab-Traub N. Consistent transcription of the Epstein-Barr virus LMP2 gene in nasopharyngeal carcinoma. J Virol 1992;66:3257–62.
16. Gilligan KJ, Rajadurai P, Lin JC, et al. Expression of the Epstein-Barr virus BamHI A fragment in nasopharyngeal carcinoma: evidence for a viral protein expressed *in vivo*. J Virol 1991;65:6252–9.
17. Hitt MM, Allday MJ, Hara T, et al. EBV gene expression in an NPC-related tumor. EMBO J 1989;8:2639–51.
18. Decaussin G, Sbih-Lammali F, De Turenne-Tessier M, Bouguermouh AM, Ooka T. Expression of BARF1 gene encoded by Epstein-Barr Virus in nasopharyngeal carcinoma biopsies. Cancer Res 2000;60:5584–8.

19. Wang D, Liebowitz D, Kieff E. An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. Cell 1985;43: 831–40.

20. Wei, MX, Ooka T. A transforming function of the BARF1 gene encoded by Epstein-Barr Virus. EMBO J 1989;8:2897–903.

21. Ooka T. Biological role of BARF1 encoded by Epstein-Barr Virus. Chapter 28. In: Robertson ES, editor. Epstein-Barr virus. Horizon Press, Annette Griffin; 2005. p. 613–30.

22. Sheng W, Decaussin G, Sumner S, Ooka T. N-terminal domain of BARF1 gene encoded by Epstein-Barr virus is essential for malignant transformation of rodent fibroblasts and activation of Bcl2. Oncogene 2001;20: 1176–85.

23. Rickinson AB, Kieff E. Fields Virology. 4th ed. In: Fields BN, Knipe DM, Howley PM, editors. Philadelphia: Lippincott-Raven Publishers; 2002. p. 2575–27.

24. Hayes DP, Brink AA, Vervoort MB, Middeldorp JM, Meijer CJ, van den Brule AJ. Expression of Epstein-Barr virus (EBV) transcripts encoding homologues to important human proteins in diverse EBV associated diseases. Mol Pathol 1999;52:97–103.

25. Seto E, Yang L, Middeldorp J, et al. Epstein-Barr virus (EBV)-encoded BARF1 gene is expressed in nasopharyngeal carcinoma and EBV-associated gastric carcinoma tissues in the absence of lytic gene expression. J Med Virol 2005;76:82–8.

26. zur Hausen A, Brink AA, Craanen ME, Middeldorp JM, Meijer CJ, van Den Brule AJ. Unique transcription pattern of Epstein-Barr virus (EBV) in EBV-carrying gastric adenocarcinomas: expression of the transforming BARF1 gene. Cancer Res 2000;60: 2745–8.

27. Danve C, Decaussin G, Busson P, Ooka T. Growth transformation of primary epithelial cells with a NPC-derived Epstein-Barr virus strain. Virology 2001;288: 223–35.

28. Wei MX, de Turenne-Tessier M, Decaussin G, Benet G, Ooka T. Establishment of a monkey kidney epithelial cell line with the BARF1 open reading frame from Epstein-Barr virus. Oncogene 1997;14:3073–82.

29. Tarbouriech N, Ruggiero F, de Turenne-Tessier M, Ooka T, Burmeister WP. Structure of the Epstein-Barr virus oncogene BARF1. J Mol Biol 2006;359:667–78.

30. Sall A, Caserta S, Jolicoeur P, Franqueville L, de Turenne-Tessier M, Ooka T. Mitogenic activity of Epstein-Barr virus-encoded BARF1 protein. Oncogene 2004;23: 4938–44.

31. Strockbine LD, Cohen JI, Farrah T, et al. The Epstein-Barr virus BARF-1 gene encodes a novel soluble colony-stimulating factor-1 receptor. J Virol 1998;72: 4015–21.

32. Cohen JI, Lekstrom K. Epstein-Barr virus BARF1 protein is dispensable for B-cell transformation and inhibits α interferon secretion from mononuclear cells. J Virol 1999;73:7627–32.

33. Miller WE, Raab-Traub N. The EGFR as a target for viral oncoproteins. Trends Microbiol 1999;7:453–8.

34. Fries KL, Miller WE, Raab-Traub N. Epstein-Barr virus latent membrane protein 1 blocks p53-mediated apoptosis through the induction of the A20 gene. J Virol 1996;70:8653–9.

35. Izumi KM, Kieff E. The Epstein-Barr virus oncogene product latent membrane protein engages the tumor necrosis factor receptor-associated death domaine protein to mediate B lymphocyte growth transformation and activate NF-κB. Proc Natl Acad Sci U S A 1997;94:12592–7.

36. Dawson CW, Rickinson AB, Young LS. Epstein-Barr virus latent membrane protein inhibits human epithelial cell differentiation. Nature 1990;344:777–80.

37. Flanagan J, Middeldorp J, Sculley T. Localization of the Epstein-Barr virus protein LMP 1 to exosomes. J Gen Virol 2003;84:1871–9.

38. Vazirabadi G, Geiger TR, Coffin WF III, Martin JM. Links Epstein-Barr virus latent membrane protein-1 (LMP-1) and lytic LMP-1 localization in plasma membrane-derived extracellular vesicles and intracellular virions. J Gen Virol 2003;84:1997–2008.

39. Dukers DF, Meij P, Vervoot MB, et al. Direct immunosuppressive effects of EBV-encoded latent membrane protein 1. J Immunol 2000;65:663–70.

40. Cheung ST, Huang DP, Hui AB, et al. Nasopharyngeal carcinoma cell line (C666-1) consistently harbouring Epstein-Barr virus. Int J Cancer 1999;83: 121–6.

41. Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C. Exosomal-like vesicles are present in human blood plasma. Int Immunol 2005; 17:879–87.

42. Raposo G, Nijman HW, Stoorvogel W, et al. B lymphocytes secrete antigen-presenting vesicles. J Exp Med 1996;183:1161–71.

43. Thery C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. Nat Rev Immunol 2002;2:569–79.