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Abstract

Epstein-Barr virus (EBV) BARF1 (BamHI-A rightward frame 1) protein is a viral oncogen and immunomodulator. BARF1 mRNA is highly and selectively expressed in nasopharyngeal carcinoma (NPC) and gastric carcinoma (GC), however, evidence for presence of BARF1 protein *in situ* is lacking. *In vitro*, the hexameric form of BARF1 is rapidly secreted (sBARF1). Secretion of BARF1 from NPC tumor cells may result in the presence of sBARF1 in sera of NPC patients.

This study describes the development of new monoclonal and polyclonal antibodies that strongly and selectively bind to 'native' NPC-derived sBARF1 protein in its natural hexameric conformation. Using these antibodies, a capture enzyme linked immunosorbent assay (ELISA) was developed to detect sBARF1 protein in sera of NPC patients. Although a detection limit of 10 ng/ml sBARF1 in serum was reached for the ELISA, only 3 of 71 NPC patients at diagnosis showed elevated levels of sBARF1 protein. Possibly sBARF1 is complexed with serum proteins shielding antibody recognition sites. However, sample denaturation prior to ELISA testing did not affect the levels observed. A MALDI-MS/MS approach was developed to overcome this problem, yielding a specific BARF1 identifier peptide. However, the sensitivity of the current setup was less than BARF1-capture ELISA. The BARF1 identifier peptide can be used to further develop a quantitative targeted proteomics approach. Using immunohistochemical staining with various anti-BARF1 antibody reagents, BARF1 could be detected in paraffin-embedded BARF1 expressing cell lines revealing a cytoplasmic localisation. Unfortunately, in patient material, no BARF1 protein expression could be demonstrated.

In conclusion, the sensitive sBARF1 capture ELISA (10 ng/mL serum) demonstrated only low levels of sBARF1 in NPC patient sera, conflicting with previously published data. A higher sensitivity of both ELISA and proteomic approach is needed for a diagnostic tool. sBARF1 remains a potential serum biomarker for NPC and GC and its diagnostic value using a targeted proteomics approach should be further evaluated.

Introduction

Epstein-Barr virus (EBV) is a human gamma-herpesvirus with B lymphocyte and epithelial cell tropism, which persistently infects about 90 % of the world population. Primary infection at early age is asymptomatic, but when infection is delayed until adolescence it may present as infectious mononucleosis. After primary infection, the virus persists latently in the host in memory B cells (1) and is rarely pathogenic in immunocompetent individuals. However, the higher risk for lymphoproliferative disease in immunosuppressed patients emphasizes that EBV persistence occurs in a tight balance with the immune system. EBV is causally linked to several carcinomas and lymphomas in immunocompetent individuals as well. EBV associates with approximately 100 % of undifferentiated nasopharyngeal carcinomas (NPC) (2-4), and about 10 % of gastric carcinoma (GC) worldwide (5-7). The EBV genome contains over 80 open reading frames, of which only a few latent genes are expressed in EBV related malignancies (8-10). EBV-positive epithelial malignancies express a viral oncogene encoded in the BamHI-A rightward frame 1 (BARF1), encoding a polypeptide of 29-33 kDa called BARF1, which is absent from EBV-positive lymphomas (6,11-14) unless viral lytic cycle is switched on (15-17).

BARF1 may drive carcinogenesis by immortalizing and transforming epithelial cells of different origin and by upregulating anti-apoptotic Bcl-2, enabling cell survival under inappropriate conditions (18-20). In addition, BARF1 can influence immune environment, and secreted BARF1 inhibits macrophage colony stimulating factor (M-CSF), thus manipulating myeloid cell growth and functions (20-23).

Although BARF1 RNA is present in a high percentage of NPC and GC cells (6,12,13), only limited evidence exist that this mRNA is actually translated into protein *in vivo*. Using BARF1 expression systems *in vitro*, BARF1 was shown to be rapidly secreted as a hexameric glycosylated protein, after cleaving of the first N-terminal 20 aa (24-26). Decaussin et al. performed SDS-PAGE Westernblot analysis of BARF1 protein expression on lysates from NPC tumor material (11). Using polyclonal antibodies directed against a BARF1 peptide (pep2: aa172–180), a band corresponding to the p31 BARF1 peptide was detected in 85 % of the undifferentiated NPC samples. Unfortunately, using NPC EBV-positive and -negative GC samples, we previously found that this Pep2 antibody showed strong signals in EBV-negative samples (27). No other research group has yet confirmed these *in situ* BARF1 findings.

The BARF1 protein may escape detection *in situ* as it appears to be rapidly secreted from the tumor cells. In a previous study, using purified NPC-derived hexameric sBARF1 protein, we detected anti-BARF1 antibodies in sera of NPC patients, although BARF1 appeared to have low immunogenicity and required native conformation for antibody binding (25). The existence of antibodies in naturally infected human subjects suggests that secreted BARF1 protein might be present in circulation and could possibly be used as a biomarker for EBV associated carcinomas. Recently, Houali et al. developed a capture enzyme linked immunosorbent assay (ELISA) and observed sBARF1 concentrations as much as 5000 ng/mL in sera (n=30) (28). In addition, BARF1 was detected in saliva of NPC patients. For this capture

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ELISA the previously mentioned pep2 antibody (11) was used as capture antibody while for detection monoclonal antibodies of our group (mouse Ab 6F4 and 4A6) was applied in combination with a denaturation step (personal communication Karim Houali). These findings have not yet been reproduced by others and the reported sBARF1 levels appear non-fysiological, as detailed elsewhere (20).

The goal of this study was to further explore the expression of BARF1 protein *in vivo*. Novel antibodies targeting 'native' NPC derived sBARF1 were developed and utilized for ELISA and immunohistochemistry (IHC). Although optimal antibody combinations were capable of detecting low levels (10 ng/mL) of sBARF1 spiked in human serum and secreted BARF1 from C666.1 NPC cells, the assay was not sensitive enough to claim presence of BARF1 protein *in vivo*. Since the antibody recognition sites on sBARF1 might be shielded by unknown serum proteins, a proteomics MALDI-MS/MS approach was evaluated, resulting in a main identifier peptide to be used in further proteomic studies. Our new antibody reagents failed to specifically detect BARF1 protein in NPC tumor tissue, although paraffin-embedded cultured cells expressing BARF1 showed specific cytoplasmic staining.

Materials and methods

Patient materials. Serum panels from histological confirmed patients with NPC were collected at the Department of Ear, Nose and Throat (ENT), Dr. Sardjito General Hospital, Yogyakarta Indonesia. NPC sera were taken prior to treatment. NPC staging was done by ENT examination and computed tomography (CT)-scan and classified according to the 1996 UICC (Union International Cancer Control) classification. Sera from EBV-positive healthy individuals were obtained from the Indonesian Red-Cross blood bank, Yogyakarta, Indonesia and the archives of the VU University Medical Center (VUmc), Department of Pathology, respectively and have been extensively characterized (29-31). EBV-negative sera were collected from healthy individuals in the Netherlands.

Tissue material used for immunohistochemistry was provided by the VUmc Pathology tissue bank. Two EBV-positive and two EBV negative NPC tumors, as well as two EBV-positive and one EBV-negative GC tumor were obtained from clinically diagnosed Dutch NPC and GC patients of which the tumors were surgically removed. EBV positivity was determined by EBER RNA *in situ* hybridisation (EBER-ISH), using PNA-probes as described before (32).

Plasmids. A consensus BARF1 DNA sequence from an Indonesian NPC patient, differing from B95.8 consensus at amino acid position 29 (valine to alanine) and 130 (histidine to arginine), was used for construction of the BARF1 expression vector pcDNA-BARF1 as previously described (25). Additionally, a Flag-BARF1 and a BARF1-Flag construct were made, with Flag tags at the N-terminus and C-terminus of BARF1, respectively.

Cell culture. 293HEK cells were cultured in Dulbecco's modified Eagle's medium (DMEM). EBV-negative NPC cell line, HONE1, was cultured in RPMI medium (Lonza) and the EBV negative gastric cancer cell line, AGS, in HAM's nutrient mixture F12 (Sigma-Aldrich) at 37ºC and 5 % CO2 in a humidified atmosphere. C666.1, a NPC cell line consistently harboring EBV

was cultured in DMEM in fibronectin-coated flasks (Sigma-Aldrich). All media contained 10 % fetal calf serum (FCS), 100 units/ml penicillin (PEN), 100 µg/mL streptomycin (STR), 2 mM L-glutamine, and 50 µM β-mercaptoethanol. After stable transfection with pcDNA-BARF1, 293HEK-BARF1 cells were cultured under selection with 95 µg/mL phleomycin (Zeocin) (Invitrogen).

BARF1 protein expression and purification. NPC-derived human secreted hexameric BARF1 (native-sBARF1) protein was produced as described previously, and purified from serum-free culture medium of stably transfected BARF1 expressing 293HEK cells using Concanavalin A beads followed by size-exclusion chromatography purification (25).

Monoclonal and polyclonal antibodies. The monoclonal antibody 4A6 was produced by immunization of mice with synthetic BARF1 C-peptide, amino acid (aa) 187 to 22 as described previously (25). Rabbit polyclonal antibodies (from now on abbreviated as pAb) to native sBARF1 was produced by immunization of a rabbit with purified native sBARF1, administered in Immacel-R adjuvant (Pickcell) as described previously (22). Rat monoclonal antibodies (from now on abbreviated as mAb #20, #31, #42 and #76) against BARF1 were produced by immunization of a rat with purified native sBARF1 followed by standard hybridoma fusion technology. Hybridomas were selected for production of antibodies detecting native sBARF1 (Figure 3A). Antibodies were purified from rabbit serum on Prot G sepharose 4 Fast Flow beads (GE Healthcare). The concentration of purified antibody was determined with a BCA protein assay kit (Pierce). Rabbit pAb nativeBARF1 and Rat mAb #42 were biotinylated (BT) with EZ-Link NHS-PEO4- biotinylation kit (Pierce) according to manufacturers instruction resulting in a Biotin-tagged antibody.

SDS-PAGE and Westernblot analysis. Cell samples were lysed in PBS containing 1 % Triton X-100 and protease inhibitor cocktail (Roche), and sonified. Cell debris was removed by centrifugation and the protein concentration was determined using a BCA Protein Assay kit (Pierce). For sBARF1 detection, the medium was harvested and debris was removed by centrifugation. For reduced SDS-PAGE, samples were diluted in 2x loading buffer with β-mercaptoethanol (BioRad) and heated for 5 min at 95°C. For non reduced SDS-PAGE, samples were diluted in 2x non-reducing loading buffer (BioRad) and incubated for 10 min at room temperature. Samples were run on a 12.5 % SDS-PAGE gel and transferred to a Hybond ECL nitrocellulose membrane (GE Healthcare). After blotting, the membrane was blocked in phosphate buffered saline (PBS) containing 3 % non-fat dry milk for 1 h at room temperature followed by incubation with 4A6 primary antibody (1:100) in PBS containing 0.05 % Tween20 (PBST) with 5 % bovine serum albumin (BSA). Anti-mouse HRP labeled secondary antibody (Dako, Glostrup, Denmark) was incubated 1:2000 in 3 % milk for 1 h, followed by 3 wash steps with PBST, and visualization by ECL Plus (GE Healthcare).

Capture ELISA. Purified rabbit pAb nativeBARF1 (1:2000) was coated onto 96-well enzyme linked immunosorbent assay (ELISA) plates (Costar) in coating buffer (0.05 M Na2CO3, pH 9.6) overnight at 4°C. Plates were washed with PBST followed by incubation with 3 % BSA in PBS for 1.5 h in 37°C to block a-specific binding sites. The next incubation steps were performed at 37°C for 1 h in sample buffer (0.05 % Tween-20, 0.1 % Triton X-100 and 1 % BSA

in PBS) per well and washing steps were performed 4 times with PBST. Samples were diluted in sample buffer and 100 µL was added to each well. Medium of BARF1 transfected cells was diluted 1:100, medium of C666.1 cells 1:25 and human serum 1:50. Purified sBARF1 (10 ng/mL) diluted in medium or human serum was used as positive control, LMP-C peptide was used as negative control. Hereinafter, wells were washed and the biotinylated detection antibody (diluted 1:10,000) was incubated. Biotin was detected through HRP-labelled streptavidin (Dako) (diluted 1:1000). For signal amplification, the catalyzed reporter deposition (CARD) method was used. Biotin-tyramide (BT) (kindly provided by W. Vos pathology VUmc) was incubated 15 min at room temperature in the dark, diluted 1:100 in sample buffer with 0.01% H2O2. After the wash steps, streptavidin/HRP (diluted 1:500) was added to the wells for 30 min at room temperature in the dark. HRP was visualized with 5'5',3'3'tetramethylbenzidine substrate reagent (TMB, BioMerieux,) for 5-15 min in the dark and reaction was stopped with 1M H2SO¬4. Optical density (OD) was measured at 450 nm with the Tecan Spectafluor. Capture ELISA using rat monoclonal antibody was performed as above with the following modifications; purified rat mAb #20 (1 µg/mL) was coated, 100 µL biotinylated detection antibody #42BT (1:5000) in sample buffer was used for detection, and TMB substrate reagent was incubated for 30 min in the dark.

Immunofluorescent staining. Cells were either cytospinned at 650 rpm for 5 min (293HEK) or grown on glass cover slides (Hone1), washed 3 times in PBS and fixed in 4 % paraformaldehyde in PBS for 10 min at room temperature. After permeabilization for 10 min in 0.25 % Triton X-100 and washing, blocking was performed with the appropriate normal serum (same animal as secondary Ab) 1:10 diluted in 3 % BSA in PBS for 15 min at room temperature. Anti-Flag mAb (Sigma F1804) 1:500 in 3 % BSA in PBS was incubated for 2 h at 37°C. Purified rabbit pAb (1:1000) and rat mAbs (1:1000) diluted in PBS with 3 % BSA were incubated for 1 h at room temperature. After three washes, FITC-labeled secondary antibodies 1:50 in 3 % BSA-PBS were incubated in the dark for 1 h at room temperature. After three washes with PBS in the dark, slides were briefly stained with 4',6-diamidino-2-phenylindole (DAPI) and Evans blue, dehydrated and mounted. IF staining were directly examined for fluorescence using a Leica fluorescent microscope, pictures were made using Leica Software.

Immunohistochemical BARF1 staining. To optimize BARF1 immunohistochemical (IHC) staining, 293HEK cells (negative control) and HEK293 cells transiently transfected with the pcDNA4-BARF1 vector with Monensin treatment (50 µg/mL, 5 h before harvest) were used. Cells were trypsinized, washed once with PBS and fixed overnight with 4 % formalin. Fixed cells were brought into a matrix using the Shandon Cytoblock Cell Block Preparation System (Thermo Scientific) according to the manufactures instructions, and embedded in paraffin. BARF1 staining of all embedded tissue was performed using the Powervision Plus method (Dako). Sections were rehydrated and endogenous peroxidise was blocked by a 30 min incubation in methanol containing 0.3 % H_2O_2. After a brief wash with water, antigen retrieval was performed by boiling the sections for 10 min in 10 mM Tris-EDTA in demi water, pH 9. Sections were allowed to cool down slowly, and were subsequently washed with tap

water and PBS. Sections were incubated for 10 min with 5 % BSA in PBS to block non-specific binding, followed by an 1 h incubation which   primary anti-BARF1 antibody diluted in antibody diluent (Immunologic) and 3 times washed with PBS. Sections were incubated for 15 min with post-antibody-blocking (Immunologic), washed 3 times and were incubated with poly-HRP-goat-α-mouse/rabbit/rat IgG (Immunologic) for 30 min, followed by 3 wash steps. Sections were stained with liquid DAB (Envision, Dako) for 10 min and excessively rinsed in water. A counterstaining with haematoxyline was conducted, and sections were washed in water, followed by a brief rinse in 1 % ammonium hydroxide. Sections were dehydrated and mounted with the Tissue-Tek Film (Sakura).

Results

The development of antibodies reactive against native hexameric sBARF1.

Previous attempts by our group to detect BARF1 *in vivo* by immunohistochemistry or ELISA were performed using antibodies developed against BARF1 synthetic peptides or purified His-tagged BARF1 produced in *Spodoptera frugiperda* (Sf9) insect cells (27). Thus far, these experiments resulted in negative outcome, although the antibodies were capable of detecting BARF1 protein by Westernblot and specifically detected BARF1 protein expressed in insect cells by immunohistochemistry. During experiments to detect anti-BARF1 antibody responses in sera from NPC patients, no responses could be found against the synthetic peptides nor to sf9 BARF1-His protein (25). In contrast, using purified NPC-derived 'native' hexameric sBARF1 antibody responses could be detected in some sera from patients with NPC (25), indicating that peptides and BARF1 produced in non-human expression systems might not be representative for the epitopes recognized *in vivo*, nor for epitopes exposed *in vivo*.

An antibody to the native hexameric conformation of BARF1 was developed in rabbit (pAb nativeBARF1). Figure 1A shows the new polyclonal compared to 4A6 monoclonal anti-BARF1 C-peptide. Both the 29 kDa band representing denatured BARF1 monomer and the lower size deglycosilated form are detected with the new polyclonal antibody. Additionally, a higher molecular weight band was detected in both the control and the BARF1-containing medium, likely to be serum albumin resulting from trace amounts of albumin contaminating the pure sBARF1 that was used for immunization. Next, the capability of the new polyclonal antibody to detect sBARF1 in its native conformation was tested by immunoprecipitation (IP). While previously none of the antibodies raised against BARF1 peptides, nor the antibody raised against sf9 BARF1-His (R150-3) were capable of precipitating sBARF1, the new polyclonal was capable of precipitating BARF1 from both medium and cell lysate (Figure 1B). Note that even while the BARF1 band in 293BARF1 lysate is barely visible because of the rapid secretion from the cells, the IP band (10-fold concentrated from lysate) is bold, indicating strong and selective binding of the protein in a low-concentration environment. Using this IP procedure, sBARF1 was shown to bind macrophage colony stimulating factor (M-CSF) (Figure 1C) (22).

Figure 1. Polyclonal antibodies binding hexameric BARF1. [A] Westernblot to compare reactivity of new rabbit polyclonal antibodies (developed against purified sBARF1) with 4A6 mouse monoclonal antibodies developed to BARF1 C-peptide (25). Medium of HEK293 transfected with (+) or without (-) BARF1 expression vector was loaded. The higher bands on the polyclonal blot are non-specific and possibly albumin. [B] Immunoprecipitations of BARF1 from culture medium and lysate using new polyclonal rabbit antibody. Before IP procedure, pre; after IP procedure, post. The following Westernblot was incubated with 4A6 antibody for detection. [C] Selection of rat hybridoma monoclonals on their performance on immunoblot and their response to sBARF1 presented by the rabbit polyclonal antibody in ELISA. [D] Immunoprecipitations, using dimethylpimilimidate-coupled pAb anti-nativeBARF1 beads, were performed with M-CSF, in the presence or absence of sBARF1. As a control, medium with both sBARF1 and M-CSF is loaded. M-CSF is specifically precipitated when sBARF1 is present.
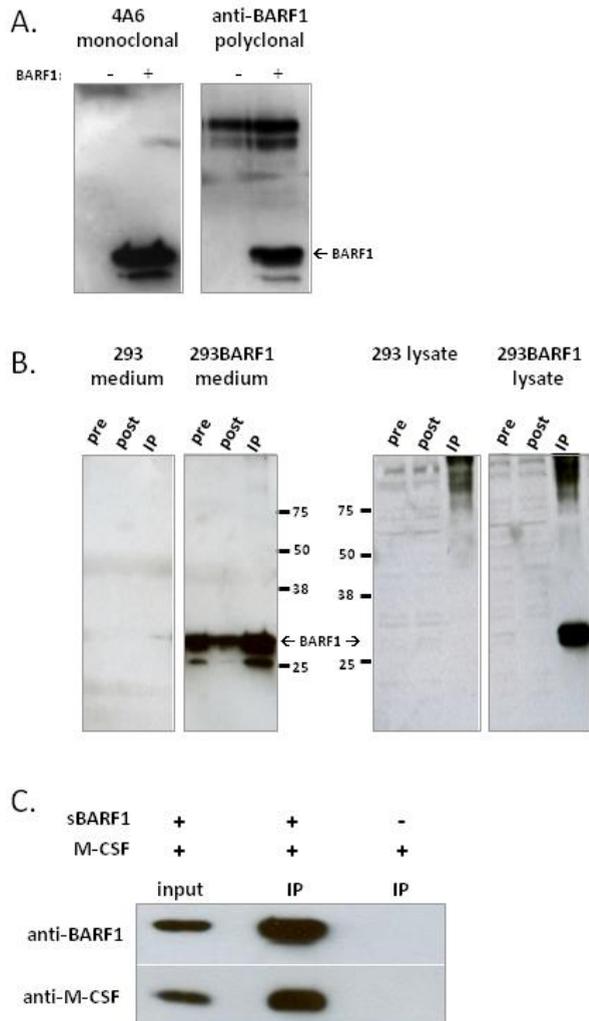
Development of a BARF1 capture-ELISA with rabbit polyclonal antibody

BARF1 protein is rapidly secreted into culture medium of BARF1 transfected cells *in vitro* which implies that the BARF1 might also be secreted from carcinoma cells *in vivo*. A suitable detection system is required to detect sBARF1 in patient samples. The new pAb native BARF1 antibody was implemented to capture and detect sBARF1 in a capture-ELISA in which the detection antibody was biotinylated. The catalyzed reporter deposition (CARD) method was used for amplification of the signal (33). Using purified sBARF1 serially diluted in human serum background, coating, blocking and sample conditions were optimized until a sensitivity of 0.125 ng per well, which reflects 62.5 ng sBARF1 per mL serum, was reached (Figure 2A).

To show that endogenous BARF1 can also be detected, the NPC cell line C666.1, which consistently carries EBV, was analyzed for BARF1 secretion. Serum free medium of C666.1 cells (harvested at 3 days of culture) was compared to a calibration curve using purified

sBARF1 in serum free medium. Quantification showed values around 125 ng/mL (Figure 2B), establishing that naturally secreted BARF1 can be measured using this ELISA system. Medium from HeLa and 293HEK cells transiently transfected with a BARF1 expression vector showed considerably higher levels around 1000 and 2700 ng/mL 2 days after transfection respectively (Figure 2C).

Secreted BARF1 protein might be present in serum of patients with NPC and could possibly be used as a biomarker for EBV associated carcinomas. Analysis of NPC patient sera (n=131) and healthy controls (n=6) normalized against EBV-negative healthy donors (n=4) revealed that most sera do not reach BARF1 levels above cut-off values. Nonetheless, 40 out of 131 NPC patient sera (31 %) gave a slightly positive reaction (Figure 2D). Using a Mann-Whitney test, a p-value of 0.23 was calculated, indicating that the difference of serum sBARF1 levels between healthy donors and NPC patients is not significant.
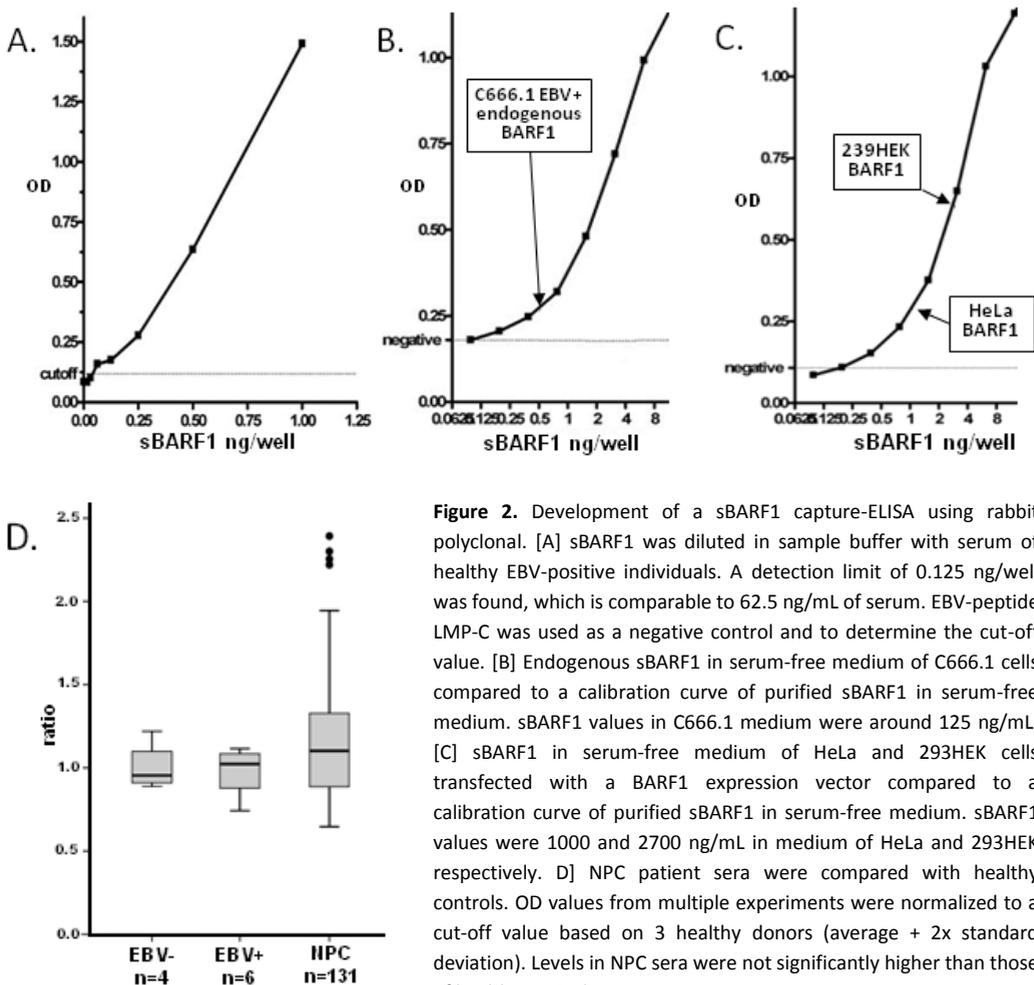

Figure 2. Development of a sBARF1 capture-ELISA using rabbit polyclonal. [A] sBARF1 was diluted in sample buffer with serum of healthy EBV-positive individuals. A detection limit of 0.125 ng/well was found, which is comparable to 62.5 ng/mL of serum. EBV-peptide LMP-C was used as a negative control and to determine the cut-off value. [B] Endogenous sBARF1 in serum-free medium of C666.1 cells compared to a calibration curve of purified sBARF1 in serum-free medium. sBARF1 values in C666.1 medium were around 125 ng/mL. [C] sBARF1 in serum-free medium of HeLa and 293HEK cells transfected with a BARF1 expression vector compared to a calibration curve of purified sBARF1 in serum-free medium. sBARF1 values were 1000 and 2700 ng/mL in medium of HeLa and 293HEK respectively. D] NPC patient sera were compared with healthy controls. OD values from multiple experiments were normalized to a cut-off value based on 3 healthy donors (average + 2x standard deviation). Levels in NPC sera were not significantly higher than those of healthy controls.
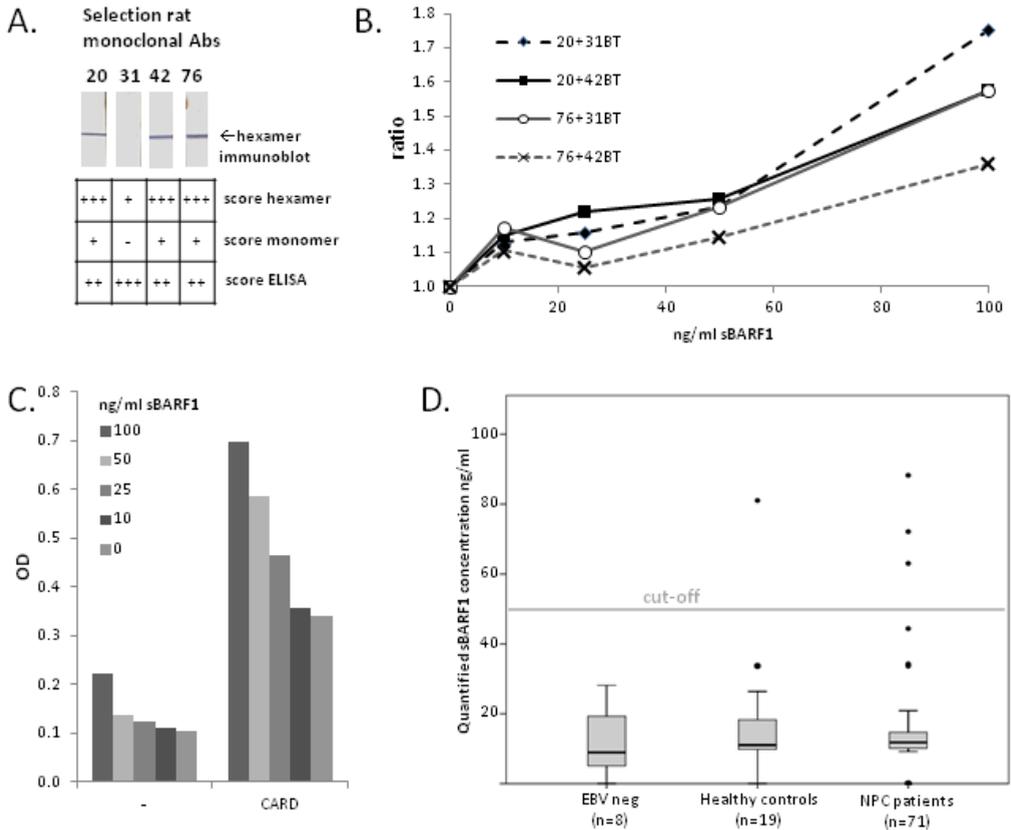
Figure 3. Development of a sBARF1 capture-ELISA using rat monoclonals. [A] Selection of rat hybridoma monoclonals on their performance on immunoblot and their response to sBARF1 presented by the rabbit polyclonal antibody in ELISA. Only the hexamer blot is shown. [B] Comparison of the four best capture-detection combinations of rat monoclonals at lower sBARF1 in serum values. Y-axis shows OD values normalized to non-spiked pool serum. Capture #20 and detection #42 was the best combination. [C] CARD enhancement of #20 capture and #42BT detection enhanced OD values. [D] Analysis of sBARF1 in sera of EBV negative controls (n=8), EBV-positive healthy controls (n=19) and NPC patients (n=71).

Development of a BARF1 capture-ELISA with rat monoclonal Antibodies

To obtain a better sensitivity and higher specificity, monoclonal rat antibodies were made. Four hybridoma clones were selected based on their ability to recognize sBARF1 presented by rabbit pAb nativeBARF1 in ELISA, as well as their responses to sBARF1 hexamer and to sBARF1 monomer by immunoblot (Figure 3A). Using serial dilutions of purified sBARF1 from a human cell expression system spiked in a serum pool of human healthy EBV-positive donors, the limit of detection of the combination rabbit pAb native BARF1 capture antibody and either of the four rat monoclonal antibodies was around 100 ng/mL sBARF1. To obtain a higher sensitivity the different capture and detection combinations of the four rat monoclonals were tested. Monoclonal #31 and #42 were purified and biotinylated and used for detection, mAbs #20 and #76 were low producers and could therefore only be tested as capture antibody. Checkerboard titration was done, systematically varying the concentration

of two antibody components, for coating and detection, finally detecting low sBARF1 concentrations in serum (10 ng/mL v.s. serum pool background). The combination of mAb#20 with the detection rat mAb #42 BT revealed the highest sensitivity and reproducibility (Figure 3B). For amplification of the signal the catalyzed reporter deposition (CARD) method was used (Figure 3C). To remove sBARF1 associating serum proteins possibly hampering detection, various mild denaturing agents such as Urea, TritonX100 and SDS were evaluated. None of these were able to improve the lower detection limit and non were implemented. Using this optimized ELISA, sera from NPC patients (n=71), EBV-positive healthy controls (n=19) and EBV-negative sera (n=8) were analyzed. A calibration curve was made by serial dilutions of purified sBARF1 in a pool serum of healthy controls (10, 25 and 50 ng/mL). For a diagnostic assay, a clear difference should exist between healthy EBV-positive donors and patients with NPC. The cut-off value, based on the mean of OD450 value of 19 healthy controls + 2x standard deviation was 52 ng/mL BARF1. Only 3 out of 71 NPC patients (4 %) were above cut-off. When based on EBV-negative sera instead of EBV-positive healthy controls, cut-off value was 33 ng/mL, resulting in 2 out of 19 (11 %) of the healthy EBV-positive controls and 7 out of 71 (10%) NPC patients reaching levels above cut-off. Figure 3D shows a boxplot of all three groups and the diagnostic cut-off level based on healthy EBV-positive controls. Three NPC patients with high sBARF1 levels were quantified by an additional standard curve ranging from 10 to 300 ng/mL, which resulted in an average of 92 ng BARF1 per mL serum. Of the 3 positive NPCs, 1 sample was taken in a Dutch hospital and this patient had distinctly high viral loads in blood and nasopharyngeal brush. Mann-Whitney analysis confirmed that sBARF1 protein levels in serum of healthy donors and NPC patients were not significantly different.

Table 1. Number of BARF1 identifying spectra for the peptide sequences they represent.

	peptide	BARF1 IP	pure sBARF1 high
1	gqavtaflger	1	2
2	(r)vslgpeievswfk	7	1
3	lgpgeeqvligr	6	13
4	mklgetevtkqehlsvvkpl	0	4
5	ndkeeahgvyvsgylsq	1	7

Proteomic analysis of BARF1

It is possible that BARF1 protein *in vivo* rapidly complexes (e.g. with M-CSF) and therefore may be shielded from antibody detection. A MALDI-MS proteomics approach would overcome this problem, so secreted BARF1 protein (sBARF1) was evaluated as a MALDI-MS/MS proteomic serum biomarker (34). ExPASy's peptidecutter was used to predict BARF1 peptides that can be obtained after trypsin digestion (35). sBARF1 has 16 potential trypsin cleavage sites, resulting in 7 potentially identifiable peptide fragments when fully digested as depicted in Figure 4A. To start, a pure sBARF1 sample, BARF1 immunoprecipitated (IP) from 293HEK BARF1 expression vector transfected cell lysate with rabbit pAb nativeBARF1, and a

BARF1 IP of control lysate were run on an SDS-PAGE gel. In-gel digestion was performed on a gel slice cut out between 20 and 30 kDa. BARF1 was positively identified in both the pure sBARF1 and the IP BARF1 lysate. When analyzing spectra of BARF1 in more detail, it was found that BARF1 was mainly identified by peptides 2 and 3, (r)vslgpeievswfk and lgpgeeqvligr, respectively (Table 1). To test whether BARF1 could be identified in lower concentrations as needed for a diagnostic setting, pure sBARF1 was diluted to 100 and 1000 ng/ml in PBS, loaded on an SDS-page gel (1 ng/gel slide and 10 ng/gel slide respectively) in-gel digested and analyzed. Unfortunately, the MS/MS assay only detected sBARF1 peptide at 1000 ng/ml initial BARF1 concentration. The detected peptide was peptide 3 (lgpgeeqvligr).

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1    marfiaqlll lascvaaqqa vtaflgerat ltsywrrvsl gpeievswfk lgpgeeqvli
                                                       1                    2              3
61   grmhhdvifi ewpfrgffdi hrsantfflv vtaanishdg nylcrmklge tevtkqehls
                                                                                  4
121  vvkpltlsvr sersqfpdfs vltvtctvna fphphvqwlm pegvepapta anggvmkekd
181  gslsvavdls lpkpwhlpvt cvgkndkeea hgvyvsgyls q
                                        5
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Figure 4. Peptide cutter analysis and MS-proteomics sBARF1 identifying peptides. BARF1 amino acid sequence is shown with bold Ts indicating the trypsin cleavage sites. The first 20 aa, predicted to be cleaved off before BARF1 is secreted are indicated in a lighter shade. Gray bars indicate the identifying peptides.

BARF1 intracellular localization by immunofluorescent staining.

Immunohistochemical detection of BARF1 protein in EBV-positive NPC and GC tumor tissue sections has been hampered by the lack of specific antibodies. To investigate whether the newly developed rabbit polyclonal or the rat monoclonal antibodies would be suitable for immunohistochemical staining, first immunofluorescence staining was performed. BARF1 expression constructs were transiently transfected in 293HEK cells. While C-terminal Fag tagged BARF1 (BARF1-Flag) was used to confirm specificity of the antibodies, N-terminal tagged BARF1 (Flag-BARF1) was used to exclude a disturbing influence of the Flag tag on localization. While most BARF1 protein is secreted into the medium within 24 hours after transfection, BARF1 could be detected in the transfected cells (Figure 5). No BARF1 staining was detected in untransfected cells. BARF1-Flag transfected cells show cytoplasmic staining, both with anti-Flag (panel B) and anti-BARF1 Rabbit polyclonal (panel A). N-terminal FLAG-tag expression shows more restricted perinuclear staining with anti-FLAG, possibly reflecting the location of the cleaved-off N-terminal fragment (panel C), Rabbit pAb native BARF1 showed similar staining patterns both in N- and C-terminal tagged BARF1, confirming that the C-terminal Flag-tag had no major impact on localization of the major aa 20 to 333 part of BARF1 (panel A and E). Rat monoclonal antibody #42 displays a similar cytoplasmic staining pattern as pAb native BARF1 (panel E). Along with 293HEK cells, also transiently BARF1 transfected AGS cells (data not shown) and Hone1 cells showed cytoplasmic staining with rabbit pAb (panel F), and clear perinuclear staining with anti-Flag (panel G).

BARF1 immunohistochemical staining

To enable BARF1 immunohistochemical (IHC) detection in EBV-positive GC or NPC tumor tissue, staining was optimized using paraffin embedded HEK293 cells transfected with pcDNA-BARF1 and subsequently treated with monensin. Wild type HEK293 cells were used as negative control. Figure 6A showed brown cytoplasmic staining in 293HEK cells expressing BARF1 but not in control cells, in agreement with the IF-staining pattern.

Two EBV-positive and two EBV-negative NPC tumor sections were stained for BARF1 using rat mAb #20. Representative pictures are shown in Figure 6B. In EBV-positive tissue, as determined by EBER-ISH, several tissue structures relating to undefined cells and fibrous material in tumor surrounding stroma seemed positive for BARF1 but tumor epithelial cells were virtually negative. Of these, morphologically distinguishable cells were identified as false-positively stained plasma cells which are notorious for being 'sticky' (Figure 6B). Lack of suitable collection of EBV-positive NPC tissues hampered further analysis.

Two EBV-positive and one EBV-negative GC tumor section were stained for BARF1 using rat mAb #42. Representative pictures are shown in Figure 5C. Similar to the NPC tissue, the positively stained tissue structures in the GC sections were not of epithelial origin, but were identified by a pathologist as healthy aspecifically stained gastric neuro-endocrine cells. We can therefore conclude that while the rat anti-BARF1 antibodies specifically stained BARF1 expressing HEK293 cells, in NPC and GC tissue samples no discrimination could be made between background staining and true BARF1 staining.

Discussion

BARF1 is a viral oncoprotein with pleiotropic functions, contributing to cell growth and survival as well as to immune modulation (20). While BARF1 mRNA is frequently detected in EBV associated carcinomas such as NPC and GC, evidence of the actual protein being translated *in vivo* is limited. Detection of BARF1 protein *in situ* could give us more insight in the fysiological role of BARF1 and could serve as a diagnostic marker for GC and NPC. A hurdle in the development of these assays is the complex double three-ring structure of the hexameric sBARF1 protein (26). This 3D structure hampers the development of antibodies by using synthetic peptides or recombinant proteins. In this study new antibodies against BARF1 were developed, capable of strong and selective binding of the hexameric BARF1 protein in its native conformation from a low concentration environment, complementing our collection of antibodies reactive with denatured BARF1. The ELISA subsequently developed with the newly developed rabbit polyclonal antibody was capable of detecting wild type sBARF1 in medium of C666.1 cells, a naturally infected EBV-positive NPC cell line, showing that our antibodies are indeed capable of detecting natural secreted wild type BARF1. However, both the IP and the detection of sBARF1 from C666.1 cells were performed in complete medium possibly complexed with serum proteins such as M-CSF that sBARF1 interacts with *in vivo*.
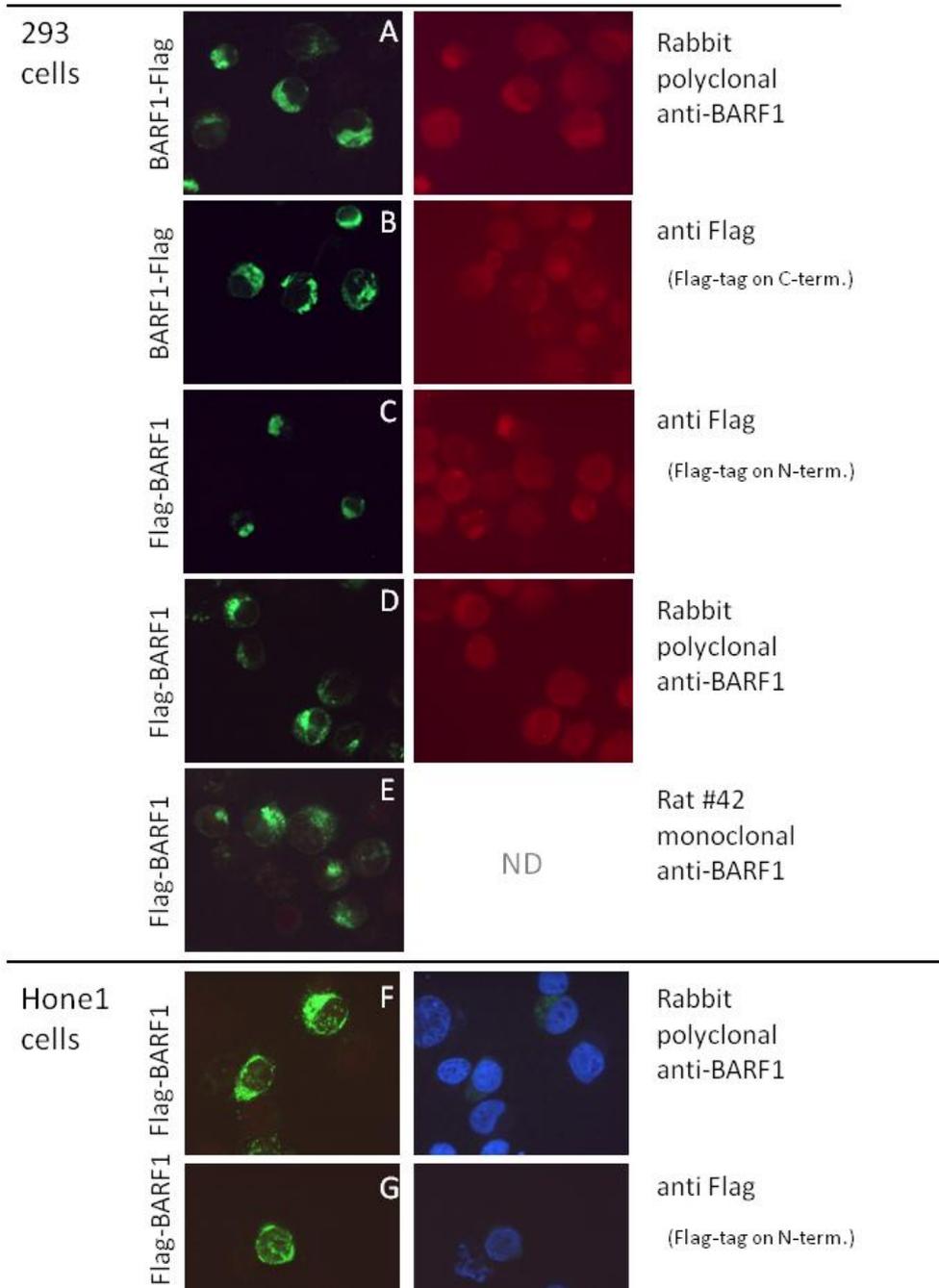
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Figure 5. BARF1 intracellular localization. Immunofluorescent staining with different antibodies shows intracellular staining of BARF1 and perinuclear localization of the N-terminal 20 aa BARF1 fragment. 293HEK cells and Hone1 cells plated on glass cover slips were transiently transfected with either Flag-BARF1 (N-terminal Flag-tag) or BARF1-Flag (C-terminal Flag-tag) and stained with rabbit polyclonal antibody anti-BARF1, Rat #42 monoclonal antibody anti-BARF1 of anti-Flag (left column). Dapi/Evans blue was used to visualize nuclei, showing both BARF1-positive and -negative cells (right column).
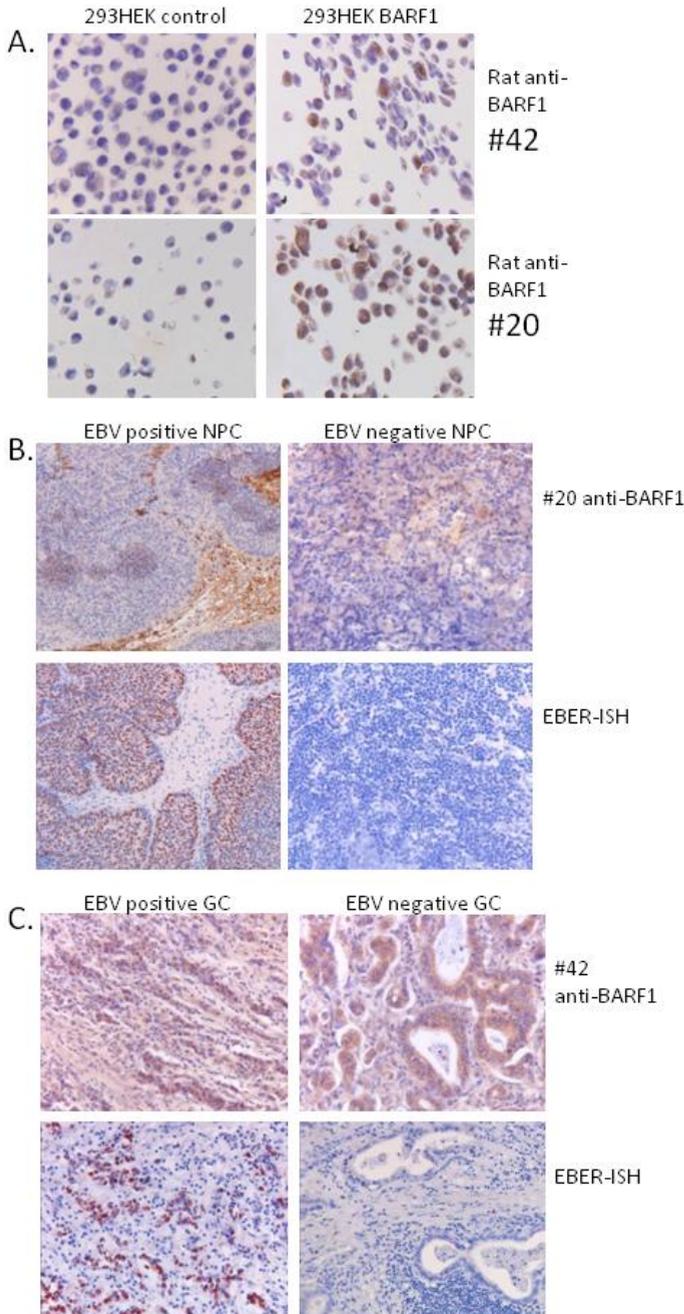
Figure 6. BARF1 immunohistochemical staining of NPC and GC tumor tissues. [A] Optimization of BARF1 immunohistochemical staining on wild type HEK293 cells (left column) and BARF1 expressing HEK293 cells treated with monensin (right column). Cells were stained with 20 ng/mL rat anti-BARF1 #20 after heat-induced antigen retrieval using TRIS/EDTA or 20 ng/mL rat anti-BARF1 #42 without antigen retrieval. Brown staining represents staining of BARF1 protein, counterstaining was performed with heamatoxyline. [B] Representative pictures of rat anti-BARF1 #20 staining of NPC tissue. EBER-ISH indicates EBV positivity. [C] Representative pictures of rat anti-BARF1 #42 staining of GC tissue. EBER-ISH indicates EBV-positivity.

Subsequently, we developed two new monoclonal antibodies targeting different conformational BARF1 epitopes. Both antibodies performed well in immunoblot and were able to detect native hexameric sBARF1 to a detection limit of 10 ng/mL in ELISA. When cut-off values were based on EBV negative sera, equal percentages of healthy EBV-positive controls and NPC patients were found positive for BARF1 (11 % and 10 % respectively), although the low numbers of healthy controls have to be taken in consideration. Since BARF1 mRNA is abundantly expressed in NPC tumors, secreted BARF1 serum levels are expected to be elevated in NPC patients. To use BARF1 protein as a diagnostic tool for NPC, the capture ELISA has to show a clear distinction between BARF1 levels in healthy donors and NPC patients. However, we found no significant difference between healthy controls and NPC patient sera. Only 3 out of 71 NPC patients showed BARF1 levels above the diagnostic cut-off with an average of 92 ng/mL. We conclude that the assay in its current form is not sensitive enough to conclusively state that sBARF1 is present in NPC patient sera at levels above those found in healthy EBV carriers. While secreted BARF1 is a likely candidate for diagnostic serum assays, thus far only one earlier report has shown presence of BARF1 in sera of patients with NPC (28). In this study (28) sBARF1 levels were found up to 5000 ng/mL in sera from patients with NPC, which were much higher than detected in the present study. A difference is within the antibodies used. Pep2 capture antibody used was reported to be aspecific (11,27) and the 4A6 and 6F4 mouse monoclonal antibodies used for detection were never successfully to bind native hexameric BARF1. Another difference was the denaturation of proteins. BARF1 possibly complexes with serum proteins *in vivo*. We were not able to improve the lower detection limit in ELISA with mild detergents, which could also mean that the association with these proteins is strong. To evade the use of antibodies and to overcome the problem of epitope shielding, the detection method of choice would be targeted proteomics. The current MALDI-MS/MS proteomics approach is not sensitive enough, but revealed a BARF1 peptide that in future studies can be used to set up a targeted proteomics approach. If BARF1 is present, as indicated by westernblot of sucrose gradient serum fractions and NPC tissue samples using Pep2 antibody (11,28) and suggested by the presence of antibodies to BARF1 (25), sBARF1 levels are likely to be below our detection limit of 10 ng/mL or present as tightly bound complexes masking available epitopes for our antibody reagents. The latter seems unlikely, however.

BARF1 might be detectable as intracellular protein in tumor sections. Immunofluorescent staining of transiently BARF1 transfected cell lines showed cytoplasmic staining of BARF1, possibly in the endoplasmatic reticulum (ER) or Golgi membrane, relating to the secretory pathway as previously described (36,37). This was not disturbed by adding either a C- or an N-terminal Flag-tag to the full length BARF1. Both rabbit pAb and rat mAbs are fully capable of detecting intracellular BARF1 in this artificial expression system and can be used in future studies to unravel BARF1 intracellular processing and colocalization. Interestingly, although only part of the cells in the transfected culture show cytoplasmic expression of BARF1, the levels of secreted sBARF1 are 100 to 1000-fold higher. The immunofluorescence data were reproduced by immunohistochemical staining of paraffin embedded BARF1 transfected cells,

which again showed specific cytoplasmic staining of BARF1. However, in EBV-positive GC and NPC tissue, apparent nonspecific coloration of stroma and infiltrating cells was seen, disturbing evaluation. Despite the good performance of our antibodies and their capability to capture native BARF1 in overexpressing cells, we were still not able to detect BARF1 *in situ* in NPC and GC tumor cells. Experiments using BARF1 expression systems showed that BARF1 is rapidly secreted into culture medium (24,25,38,39). It is likely that endogenous expression with lower levels of protein goes even more efficient, leaving undetectable amounts of intracellular BARF1. We hypothesize that BARF1 is rapidly and fully secreted from tumor cells, directly binding to M-CSF or other yet unknown factors to form complexes that are rapidly degraded by local myeloid cells in the tumor micro-environment, thus remaining undetectable for antibody staining. This agrees with the immunological properties of BARF1 in humans, being properly cross-processed for T cell epitopes (40), while being low or non-immunogenic for humoral responses (25,41). Further evaluation and quantification of sBARF1 *in vivo* as carcinoma-specific diagnostic marker is needed, but also to establish its role as an oncoprotein. Targeted proteomics might help to detect complexed BARF1 protein and coimmunoprecipitation experiments with either Flag-tagged BARF1 or with the use of antibodies might reveal which serum proteins may be involved.

Acknowledgements

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