Epstein-Barr virus encoded BARF1 protein: immunopathogenic role, transcriptional regulation and diagnostic use in nasopharyngeal carcinoma

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VARIOUS
GLYCOSYLATION
IMMUNODOMINANT

Summary

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The Epstein-Barr virus (EBV) is strongly and causally associated with two malignancies of epithelial origin, namely almost 100 % of undifferentiated nasopharyngeal carcinoma (NPC) and 10 % of all gastric carcinoma (GC) worldwide. EBV shows a latency gene expression profile in these tumors, meaning that only a few EBV genes are expressed that are essential for tumor cell growth and survival and viral persistence. One of these genes is the BamH-I rightward frame 1, known as BARF1 which is the main topic of this thesis. BARF1 is a viral oncogene with pleiotropic functions, contributing to cell growth and survival as well as to immune modulation. Interestingly, BARF1 is selectively expressed in EBV associated carcinomas and not in lymphomas, indicating that its role might differ depending on cell type. More insight in this role might support the development of treatment options for EBV associated cancers. Beside its usefulness for the virus and for the tumor, BARF1 has specific features that potentially have diagnostic implications, the most important being that BARF1 is a secreted protein, opening options for less invasive sampling and screening.
The aim of this thesis was to further investigate the function, regulation and diagnostic use of the BARF1 protein in EBV associated carcinomas.

Chapter 2 includes a state-of-the-art review about BARF1. Deciphering the role of BARF1 in EBV biology will contribute to novel diagnostic and treatment options for EBV driven carcinomas. Starting with a description of its protein structure and expression in EBV associated malignancies, the aspect of BARF1 as a diagnostic tool is also briefly touched. The oncogenic potential and immune modulating properties of BARF1 are discussed in detail, including indications of aspects that need further research.
EBV-positive nasopharyngeal carcinoma cells express the viral BARF1 gene, which encodes a secreted hexameric protein. Our aim was to analyze if serum of patients with NPC contains antibodies against BARF1 protein, to use these for early diagnosis of NPC.
Chapter 3 describes the development of a serological assay to detect antibodies to BARF1 protein. Previous work by our group (2001-2005; VUmc thesis J. van Beek) showed that short peptides from predicted immunodominant domains of the BARF1 protein and the recombinant whole protein purified from non-human expression systems provide suitable tools for the analysis of immune responses. While antibodies could be generated against these peptides and recombinant proteins in mice and guinea pigs, capable of recognizing denatured BARF1 from a human expression system, human antibodies were not detected with these tools. Since this could be due to the more recently defined (2006) complex hexameric structure of BARF1, we expressed and purified native NPC derived BARF1 from a human expression system in which posttranslational modifications such as glycosylation and folding resembles the secreted BARF1 protein *in vivo* (sBARF1). Using this purified native hexameric sBARF1 protein we could show that NPC patients have significantly higher antibody responses to sBARF1 compared to healthy volunteers, however the anti-BARF1

responses were relatively weak compared to the responses to other EBV proteins, such as VCA-p18 and EBNA1. The presence of antibodies against native BARF1 in patients with NPC indirectly proved that BARF1 may circulate *in vivo* as a hexameric protein released by tumor cells in these patients. However, due the limited immunogenicity of BARF1, this assay is not suitable as a diagnostic assay for early detection of NPC.

Sequence variation in the EBV genome can influence disease development and progression and cause changes in the interactions between the virus and the host immune system, which has been demonstrated for various key EBV genes such as EBNA1, LMP1 and the lytic switch protein Z. In **Chapter 4** the sequence variation of the BARF1 gene was investigated, in relation to EBV genotype, viral load and serology markers. The BARF1 gene from Indonesian donors shows minor single nucleotide variation with the prototype EBV B95.8 sequence, but most mutations did not result in amino acid substitutions at the protein level. The three most frequently detected amino acid changes (V29A, W72G, H130R) were not predicted to lead to gross tertiary structure alterations based on the BARF1 crystal structure. The evolutionary well conserved sequence implicates that BARF1 has functional relevance in EBV biology. Utilizing the immunoserology assay from chapter 3, we determined that the presence of amino acid substitutions in the BARF1 protein in NPC patients did not influence the detection of antibody responses to BARF1.

EBV persists lifelong under a dynamic balance with the human immune system and, like many other persistent herpes viruses, has acquired numerous mechanisms for subverting or evading immune surveillance. In **Chapter 5** the role of secreted BARF1 protein as an immune modulator was studied. The BARF1 protein has partial sequence homology with a conserved structural domain found in various growth factor receptors, one of which is the receptor for macrophage colony stimulating factor (M-CSF receptor). Utilizing our purified native sBARF1 from chapter 2, polyclonal antibodies were developed which were used to coimmunoprecipitate M-CSF together with sBARF1, demonstrating that sBARF1 is capable of capturing M-CSF. The cytokine dependent human myeloid MUTZ3 cell line was deployed to demonstrate that sBARF1 binding to M-CSF functionally interferes with cell proliferation. Antibodies specific to hexameric native sBARF1 were able to oppose this effect. From the amount BARF1 protein needed to inhibit the M-CSF action we could determine that one sBARF1 hexamer binds three M-CSF dimers. Site directed mutation of the predicted sBARF1/M-CSF interaction site revealed that sBARF1 binds M-CSF using three 'tweezer' like loops, located on the exterior of the rings. The effect of sBARF1 on M-CSF driven mononuclear phagocyte differentiation was functionally evaluated, revealing that sBARF1 exposure during differentiation negatively affected the ability to phagocytose apoptotic cells, produce oxygen radicals, and functional surface markers such as the Fc receptor CD16. We conclude that sBARF1 protein is a potent decoy receptor for M-CSF, hampering the function and differentiation of mononuclear phagocytes. BARF1 suppresses M2 macrophage differentiation, which are the first line of anti-viral defense. We hypothesize that inhibition of mononuclear phagocytes by sBARF1 decreases anti-viral defense during lytic replication, which might allow macrophages to act as virus transmission vehicle of internalized virus,

opposed to inactivation of the virus. In EBV associated malignancies BARF1 immune modulation of mononuclear phagocytes might constitute a possible mechanism by which macrophage-mediated anti-tumor immunity is evaded.

The transcriptional regulation of the BARF1 gene has been an unexplored area so far. While BARF1 mRNA can be detected in all cell types during lytic replication, in latency the gene is only expressed in tumors with an epithelial cell background, indicating that activation of the BARF1 promoter is cell type specific. In **Chapter 6** and **Chapter 7** we set out to unravel the transcriptional regulation of BARF1. EBV regulates many of its genes by promotor methylation. A bisulfite sequencing analysis showed that the BARF1 control region is highly methylated in multiple cell types as well as in C15 and C17 mouse xenograft NPC tumor material.

In **Chapter 6** a BARF1 promoter luciferase construct was created and utilized to evaluate the transactivating activity of the immediate-early proteins R and Z. Methylation of the construct enabled us to examine how methylation affects its ability to be activated. We found that the EBV encoded transcriptional activator R , but not Z, induced 50- to 250-fold upregulation of luciferase activity. Chromatin Immune Precipitation (ChIP), Electrophoretic Mobility Shift Assay (EMSA) and specific mutagenesis of the R responsive elements (RREs) demonstrated direct binding of the R protein to RREs between -554 and -327 nucleotides relative to the BARF1 transcriptional ATG start site. Using quantitative reverse transcriptase (RT)-PCR we showed that R is capable of transactivating the BARF1 promoter in the context of the viral genome, within 6 hours after transfection with a R expression vector.

In **Chapter 7** transcription factor binding site analysis of the BARF1 promotor area revealed that it contains potential binding sites for the p53 family of transcription factors. One member of the p53 family of transcription factors is the ΔNp63α isoform of p63, which is highly expressed in undifferentiated NPC and is regarded a marker for epithelial differentiation. Reporter assays performed with the BARF1 promoter luciferase construct revealed that specifically the ΔNp63α isoform, and none of the other p53 family members is capable of transactivation of the BARF1 promoter. We concluded that although ΔNp63α can transactivate the BARF1 promotor reporter construct, additional factors are required to transactivate BARF1 in the context of the viral genome. We concluded that in EBV-positive NPC and GC, BARF1 expression might be induced by the epithelial differentiation marker ΔNp63α.

While BARF1 mRNA is frequently detected in EBV associated carcinomas such as nasopharyngeal and gastric carcinoma, evidence of the actual protein being translated *in vivo* is limited. In **Chapter 8** we developed and evaluated different methods for the detection of BARF1 protein. New monoclonal and polyclonal antibodies capable of recognizing and capturing hexameric native sBARF1 protein were developed and used to set up an antigen-capture enzyme-linked immunosorbent assay (ELISA) system for detection of sBARF1 in sera of patients with NPC. The same antibodies were assessed for immunofluorescence (IF) and immunohistochemistry (IHC) in cells and tissues. BARF1 was also evaluated for its potential as a serum biomarker using a Mass-Spec MicroSequencing (MS/MS) proteomic approach,

revealing a main identifier peptide suitable for a targeted proteomic analysis. We concluded that the capture ELISA in its current form can detect sBARF1 levels above 10 ng/ml, which appeared not sensitive enough to conclusively state that sBARF1 is present in NPC patient sera at levels above those found in healthy EBV carriers. The MS/MS proteomic approach in the current format available to us at VUmc proved even less sensitive than the capture-ELISA. We further demonstrate that antibodies suitable for detecting BARF1 protein in transfected cells cannot detect BARF1 protein expression using immunohistochemistry of NPC and GC tissue, that express BARF1 mRNA. 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. These complexes are either rapidly degraded by local myeloid cells, or shield BARF1 epitopes, with the consequence that BARF1 protein can not be traced or eludes antibody capture. We suggest that future research should further focus on a targeted proteomics approach.