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A recombinant polypeptide of the megakaryocyte potentiating factor is a potential biomarker in plasma for the detection of mesothelioma

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ABSTRACT

Malignant mesothelioma (MM) is a fatal disease mostly associated with asbestos exposure and difficult to detect by non-invasive methods. This study aimed to use recombinant fragments of the megakaryocyte potentiating factor (MPF) for the development of cost-effective MPF ELISAs. Three polypeptides spanning the MPF region (MPF_{1-148}, MPF_{34-288}, $MPF/MSLN_{254-400}$) were produced in *E.coli* as maltose-binding protein hybrids. After isolation, Factor Xa digest, and purification, the polypeptides were used for the generation of rabbit antibodies and development of ELISAs. Forty-one MM patients with known histological subtype before tumor-specific treatment and 70 asbestos-exposed individuals free of any cancer were matched according to age, gender, and smoking. Plasma of all subjects was tested with the three newly developed polyclonal antibody-based ELISAs and a commercial mesothelin assay (MESOMARK™). The latter differentiated patients (median concentration 1.95 nM) from controls (median 1.07 nM, $p < 0.0001$) and showed an area under curve (AUC) of 0.77 in receiver operating characteristics (ROC) analysis. Of the MPF variants, exclusively the ELISA based on antibodies against the MPF_{34-288} fragment displayed significantly ($p = 0.0002$) higher values in patients than in controls (median 1.61 nM versus 0.88 nM; AUC = 0.72). The combination of the MPF_{34-288} and mesothelin displayed an improved ROC performance (AUC = 0.80). The MPF_{34-288} ELISA could be a cost-effective and minimal-invasive contribution to support a diagnosis of mesothelioma, especially in regions with a limited medical care.

1. Introduction

Malignant mesothelioma (MM) is a fatal disease associated with asbestos exposure as key risk factor [1,2]. Because the treatment options are particularly limited in advanced disease, an early diagnosis combined with a multimodal therapeutic approach may be helpful to improve the survival rate as reported for MM of the epithelioid subtype [3,4]. Although there is an urgent need for non-invasive detection methods, the availability of such markers is scarce [5]. One of the best available markers are the soluble mesothelin-related peptides (SMRP), also known as mesothelin [1,6,7]. Several efforts were made to investigate other proteins of the mesothelin family and to study their potential as MM biomarkers. One of them is the megakaryocyte potentiating factor (MPF) also known as N-ERC/mesothelin [8]. Both, mesothelin and MPF are products of the mesothelin gene *MSLN*, encoding a 71-kDa precursor protein which is cleaved by furin-like proteases into two fragments. The 31-kDa soluble N-terminal MPF fragment is secreted into blood, the C-terminal 40-kDa fragment remains membrane-bound and is classified as mature mesothelin [9–11].

The first ELISA developed by Shiomi et al. [11] was based on the monoclonal antibody (mAb) 7E7 and the polyclonal antibody (pAb) 282 directed against N-ERC/mesothelin. Subsequently, they improved this assay by replacing the pAb by the newly generated mAb 16K16 [12] and finally by mAb 20A2 [13]. In addition, two other research groups developed MPF assays using other generated pairs of monoclonal anti-MPF antibodies [14,15]. Due to its solubility and physiological origin, MPF was considered to be a promising marker to detect MM. The performance of the different MPF assays was evaluated also by other groups, mostly in comparison

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with the "gold standard" SMRP, and depended on the antibodies used [16–18].

Because the availability of cheap MPF assays is limited, we decided to generate our own pAbs as the basis for an affordable immunoassay suitable for screening of a large cohort of asbestos-exposed workers. Different MPF peptides, based on three fragments spanning the entire *MPF* coding region of the *MSLN* gene, were used to obtain pAbs for the development of three different MPF ELISAs. The purpose of this study was to evaluate the ability of the three ELISAs to discriminate between MM patients and controls.

2. Materials and methods

2.1. Patients and controls

Patients with diagnosed malignant mesothelioma (MM) were recruited at the "HELIOS Clinic Emil von Behring", Berlin, Germany. None of the patients was operated or treated by chemotherapy or radiation therapy prior to blood collection. The patient group comprised 41 persons (33 males and 8 females, mean age 69 years, range: 35–85 years). According to histological analyses, 31 of them had epithelioid, six biphasic, and four sarcomatoid mesothelioma.

The control group included 70 asbestos-exposed individuals with and without benign asbestos-related diseases like pleural plaques or asbestosis but without any evidence of mesothelioma or other cancers matched according to age, gender, and smoking status to the MM patients. Controls were frequency-matched to cases by age in 5-year groups, using the following intervals: ≤45, 46–50, 51–55, 56–60, 61–65, 66–70, 71–75, 76–80, 81–85 years.

The study was approved by the ethics committee of the Ruhr-University Bochum (reference number: 3217-08). All individuals gave written informed consent for their participation.

2.2. Blood samples

Peripheral blood was collected in S-Monovette EDTA gel tubes (Sarstedt, Nümbrecht, Germany). Within 30 min after blood collection, samples were centrifuged at 2,000×*g* for 10 min and the upper plasma phase was immediately frozen and stored at −20 °C.

2.3. MPF antigen preparation

Total RNA (0.1 mg) from HeLa cells (cervical adenocarcinoma, ATCC entry CCL-2) were obtained from Yorkshire Bioscience Ltd, York, U.K. The cDNA synthesis was performed with 2 μg total RNA in a total volume of 20 μl using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Germany). Aliquots of the cDNA were used to amplify independently three MPF fragments corresponding to amino acid residues 1–148, with primer pair MPF1_FspI_F (5′-TGCGCAATGCCAAC-GGCTCGA-3′) and MPF148_HindIII_R (5′-CAAGCTTCAATT-GGCCTTCGTGAT-3′), to amino acid residues 34–288 with primer pair MPF34_AfeI_F (5′-AGCGCTTCGA-GGACCCTGGCTGGAGAG-3′) and MPF288_HindIII_R (5′-TAAGCTTCAGAT-GGTCCGTTCGGA-3′), and to amino acid residues 254–400 with primer pair MPF254_SmaI_F (5′-CCCGGGCTGGGCCAG-CCCATC-3′) and MPF400_HindIII_R (5′-GAAGCTTCATTCAAGCAAAGCCTTCAG-3′). The PCR reaction contained 3 μl cDNA, 20 pmol of each of the primers per pair, 5 μl dNTPs, 5 μl 10× PCR buffer yielding a final concentration of 1.5 mM $MgCl_2$, and 2.5 units of Taq polymerase in a final volume of 50 μl. PCR conditions for all three constructs included an initial denaturation step at 95 °C for 5 min, followed by denaturation at 95 °C, annealing at 56 °C, and elongation at 72 °C for 1 min each for a total of 40 cycles. After a final elongation step at 72 °C for 10 min an aliquot of the PCR-reaction was analyzed by agarose gel (1.8%) electrophoresis.

All three amplification products were sub-cloned into the pDrive system (Qiagen, Hilden, Germany) for sequence analysis. One DNA clone of each fragment was chosen to generate the target fragment comprising MPF amino acid residues 1–148, 34–288, and MPF/MSLN 254–400. The gel-purified fragments were cloned into the *Xmn*I-*Hind*III restricted pMALc2 expression vector (New England Biolabs GmbH, Frankfurt, Germany) to express three different MPF target fragments in frame at the C-terminus of the 42.7-kDa carrier maltose-binding protein (MBP), necessary for the purification by affinity chromatography. The resulting MBP-MPF hybrids were purified with an amylose resin (New England Biolabs GmbH) as described [19]. Aliquots of the isolated fusion proteins were specifically restricted by Factor Xa to cleave the MPF target from the MBP carrier and separate the fragments on a 4–16% Novex gel. After staining the gel with 0.1 M KCl for 30 min at 4 °C the target protein band was cut out under incident light on a dark underground, homogenized, and eluted using the EzWay™ PAG protein elution kit (KOMABIOTECH, Seoul, Korea). The eluate was collected and an aliquot was run with a defined amount of broad range marker (New England Biolabs) on a 10% SDS-PAGE to calculate the concentration by scanning the appropriate gel slots after Coomassie staining with a GS-800 Calibrated Densitometer (Bio-Rad Laboratories GmbH, Munich, Germany). About 100 pmol of the target protein was blotted on a nylon membrane for analysis of the first five N-terminal amino acid residues by an Applied Biosystems Procise Sequencer using the Dansyl-Edman method. The remaining protein targets were collected and used for antibody generation.

2.4. Generation of polyclonal antibodies against different MPF variants

Immunization of a rabbit with purified recombinant MPF (1 mg each of $MPF_{1\text{-}148}$, $MPF_{34\text{-}288}$, or $MPF/MSLN_{254\text{-}400}$) was conducted by Charles River (Kisslegg, Germany) exactly in accordance with the standard protocol of the company. Antisera were received 72 days after primary immunization. Sera containing anti-MPF pAbs were loaded via FPLC (Thermo Fisher Scientific, Uppsala, Sweden) on a protein G column (GE Healthcare, Munich, Germany), washed with 20 mM phosphate buffer pH 7.0 and eluted with 0.1 M glycine-HCl buffer pH 2.8. Eluted fractions were neutralized directly with 1/10 vol 1 M Tris-HCl pH 9.0, pooled and dialyzed in phosphate-buffered saline (PBS) pH 7.4. Protein concentration was determined by Bradford protein assay (Bio-Rad Laboratories GmbH) with bovine serum albumin (BSA) as a standard and yielded an antibody concentration of 0.841 mg/ml (anti-$MPF_{1\text{-}148}$), 1.1 mg/ml (anti-$MPF_{34\text{-}288}$), and 1.73 mg/ml (anti-$MPF/MSLN_{254\text{-}400}$). One part of each of the purified pAbs was used later as capture antibodies. Another part was biotinylated by mixing the antibodies with a 33-fold molar excess of biotin-N-hydroxysuccinimide ester (Roche Diagnostics GmbH, Mannheim, Germany), dissolved in dimethylsulfoxid and incubated under continuous agitation for 4 h at room temperature (RT). The biotinylated antibodies were dialyzed extensively with PBS. Capture and biotinylated antibodies were stored in aliquots at −80 °C until use.

2.5. Measurement of mesothelin

Mesothelin in plasma samples was measured using the ELISA kit MESOMARK™ (Fujirebio Diagnostics, Inc., Malvern, PA, USA).