

Confirmation of the biological significance of transthyretin as a biomarker for cutaneous T-cell lymphoma by its protein interaction partners

NIKO ESCHER¹, ROBERT KOB¹, MARTIN KAATZ², CHRISTIAN MELLE¹,
CHRISTINA HIPLER² and FERDINAND VON EGDELING¹

¹Core Unit Chip Application (CUCA), Institute of Human Genetics;

²Department of Dermatology and Allergology, University Hospital Jena, 07740 Jena, Germany

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Abstract. We previously identified transthyretin (TTR) and its posttranslational modifications as a down-regulated marker in *mycosis fungoides* (MF), a benign subtype of cutaneous T-cell lymphoma (CTCL). In order to more precisely understand the biological role of TTR in the etiology of MF, it is essential to clarify the pathways of progression by identifying further interacting proteins. This study is the first to combine blue native polyacrylamide gel electrophoresis (BN-PAGE) with surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) to detect new TTR interaction partners and to determine whether these TTR interaction partners can themselves be used as biomarkers. By this procedure, apolipoprotein A1, which was additionally found to be down-regulated in the serum of MF patients, apolipoprotein A4, retinol binding protein 4 (RBP-4), and retinoid X receptor β (RXR- β) were identified as interaction partners of TTR. The RXR family plays a role in cell differentiation and proliferation and is known to be the target of bexarotene, which is used in the treatment of CTCL. In conclusion, the combination of BN-PAGE and SELDI-TOF-MS used in this study allowed for the detection of protein interaction partners, which, in the case of RBP-4 and RXR, indicated a connection between the common tumor marker TTR and tumor progression in CTCL.

Introduction

Mycosis fungoides (MF) is a subtype of cutaneous T-cell lymphoma (CTCL) and is, at a proportion of approximately

50%, the most common disease in this group, followed by CD30⁺ lymphoproliferative disorders and Sézary syndrome (1). MF typically presents in middle adulthood and is clinically divided into patch, plaque and tumor stages (2). Approximately 25% of MF patients with extensive patches or plaques develop progressive disease. To date, only a few biomarker candidates have been identified for MF. In addition to neopterin, β 2-microglobulin and soluble IL-2 receptor have been described as possible candidate markers that are elevated in CTCL patients (2,3).

In our most recently reported study, we detected and identified transthyretin (TTR) and its modifications as down-regulated biomarkers in the serum of MF patients (4). At present, TTR is known to be the major carrier for serum thyroxine (T₄) and triiodothyronine, and is also found in cerebrospinal fluid (5). The transport of retinol (vitamin A) via its interaction with retinol binding proteins (RBPs) is also facilitated by TTR. Nevertheless, it is important to identify further biomarkers that may be useful for the diagnosis of CTCL patients.

Currently, biomarkers are mainly detected or identified by an undirected search of a patient's serum, blood cells or tissue (6-9). Another hitherto unused method to find novel biomarkers is the identification of protein interacting partners of an established biomarker. This is of particular significance, since nearly all proteins have multiple interaction partners, and the up- or down-regulation of a protein in a complex protein network is likely to affect its interacting proteins.

Currently, protein-protein interactions are studied by techniques including yeast two-hybrid screening, affinity chromatography and immunoprecipitation (10,11). Using these techniques, the purification of protein complexes is either adapted to capturing the target using antibodies or to indirect immobilization through tagged proteins. For immunoprecipitation, it is necessary to have an antibody with a high affinity and sensitivity. Furthermore, the epitope may be hidden in a protein complex or block the binding of an interaction partner, which might play an essential role in the biological function of the complex. It is therefore necessary to use a technique that circumvents these limiting factors.

Correspondence to: Professor Ferdinand von Eggeling, Institut für Humangenetik, CUCA, Universitätsklinikum Jena, 07740 Jena, Germany
E-mail: fegg@mti.uni-jena.de

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Blue native polyacrylamide gel electrophoresis (BN-PAGE) was originally described by Schagger *et al* (12), and allows for the separation of protein complexes in an initial native dimension, followed by the separation of interacting proteins in a second denaturing dimension. This technique has previously been used for the analysis of murine intestinal brush border membranes, chloroplasts and membrane proteome analysis of the green sulfur bacterium *Chlorobium tepidum* (13-15). Interaction partners found with BN-PAGE may be ideally verified and identified with surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), which combines on-chip processing of biological samples with mass spectrometry, and is most commonly used for the detection of biomarkers in body fluids or tissues (8,16). However, a few studies identifying new protein-protein interactions using this technique have also been reported (11,17,18).

Combining BN-PAGE and SELDI for the first time, we found apolipoprotein A1, apolipoprotein A4, retinol binding protein 4 (RBP-4) and fragments of retinoid X receptor β (RXR- β) to be TTR interaction partners. By comparing these TTR interaction partners with a list of differentially expressed proteins identified in previous studies (unpublished data), apolipoprotein A1 was determined to be a down-regulated biomarker in the serum of MF patients.

Methods

BN-PAGE. For the first dimension, serum was diluted 1:2 with native PAGE sample buffer [50 mM bis-Tris, 50 mM NaCl, 10% (w/v) glycerol, 0.001% Coomassie Brilliant Blue G-250, pH 7.2 (Serva)]. BN-PAGE was performed using a gradient gel (4.5-15%) with a specific running buffer (50 mM bis-Tris, 50 M tricine, pH 6.8) plus 3 ml cathode buffer (running buffer containing 0.4% Serva Blue G). Electrophoresis was conducted at 130 V until the ion front migrated to the gel bottom (Fig. 1A).

For the second dimension, specific complexes from two equivalent lanes were excised from the stained gradient gel. These pieces were transferred into reaction tubes and covered with equilibration buffer (1% SDS and 1% β 2-mercaptoethanol). Subsequently, the gel bands were incubated at 95°C in SDS Laemmli buffer for 5 min, and then transferred onto two SDS gels (Fig. 1B). Electrophoresis was conducted at 180 V, then one gel was used for Western blot analysis of TTR to determine which lane contained the separated TTR complex. Finally, the corresponding lane of the second-dimension gel was stained with Coomassie Brilliant Blue G-250. The resulting bands were excised from the gel and digested with trypsin, and the peptides were analyzed using gold ProteinChip arrays and SELDI-TOF-MS (Fig. 1C).

Tryptic digestion. The protein bands were transferred into reaction tubes and incubated in 50% methanol with 10% acetic acid for 2 h. Subsequently, the gel pieces were passed through a graded series of buffers for dehydration. The dehydrated pieces were incubated with 20 ng/ml trypsin overnight at 37°C, and the resulting digest was applied to N20 ProteinChips and analyzed using SELDI-TOF-MS. The resulting spectra were interpreted using a search of the ProFound database (<http://prowl.rockefeller.edu>).

Coimmunoprecipitation of the TTR-apolipoprotein A1 complex. For coimmunoprecipitation (CoIP), protein A-agarose was incubated with 5 μ l anti-TTR antibody (anti-human prealbumin, goat) (Sigma) for 45 min. Subsequently, the beads were washed and 50 μ l of human serum was added. After a 45-min incubation, bound proteins were eluted using 15 μ l SDS buffer (40 mM Tris-HCl, pH 6.75, containing 4% SDS, 10% β 2-mercaptoethanol, 40% glycerol and 0.002% bromphenol blue) at 95°C for 5 min. The eluted proteins were loaded onto a 12% SDS polyacrylamide gel, separated by electrophoresis under 160 V running conditions, and then blotted using 150 mA for 2 h. Subsequently, the protein-blot membrane was washed, non-specific binding sites were blocked using 3% milk powder, and the treated membrane was incubated with anti-apolipoprotein antibody (goat, polyclonal) (Abcam) overnight. A secondary antibody (anti-goat) conjugated to alkaline phosphatase was incubated with the Western blotting membrane for 3 h.

Immunodepletion. Approximately 10 μ l of protein A-agarose was washed with CoIP buffer (20 mM HEPES containing 0.1 mM EDTA and 50 mM KCl). Anti-apolipoprotein A1 antibody (3 μ l goat, polyclonal) was coupled to the protein A by incubation with the agarose conjugate at 4°C for 45 min. After blocking with 3% milk powder, the protein A-agarose was washed with CoIP buffer and 4 μ l of 1:50 diluted serum was added. The supernatant was removed and applied to a copper sulfate activated IMAC 30 ProteinChip array. A negative control using a non-specific IgG antibody was similarly treated.

Results

Identification of TTR protein interaction partners. In previous studies, we demonstrated that TTR and its modifications were differentially expressed in the serum of patients with MF compared to a healthy control group (4). To assess the function of TTR and to detect new biomarkers, TTR interaction partner experiments were performed using a combination of BN-PAGE and SELDI-TOF-MS as follows: serum was loaded onto a native gradient gel (first dimension) at different concentrations. Nine bands from two equally loaded lanes were excised and transferred to nine wells of two SDS polyacrylamide gels (second dimension). The first gel was blotted onto a membrane and a specific band representing TTR was detected in one lane. Subsequently, this section of the second SDS-gel was stained with Coomassie Brilliant Blue G-250. All bands were excised and transferred to individual vials for tryptic digestion. The resulting peptide mass fingerprints analyzed by SELDI-TOF-MS were used in a search of the ProFound database. The proteins identified were TTR and four TTR interaction partners, namely apolipoprotein A1, apolipoprotein A4, RBP-4 and RXR- β . Finally, a list of differentially expressed proteins identified in previous studies using ProteinChip profiling was consulted, and a protein with a molecular mass of 28.1 kDa was found to be down-regulated in the serum of MF patients (Fig. 2).

Coimmunoprecipitation. To confirm the BN-PAGE finding that TTR and apolipoprotein A1 are complexed in human serum, a CoIP study was conducted as follows: specific anti-TTR

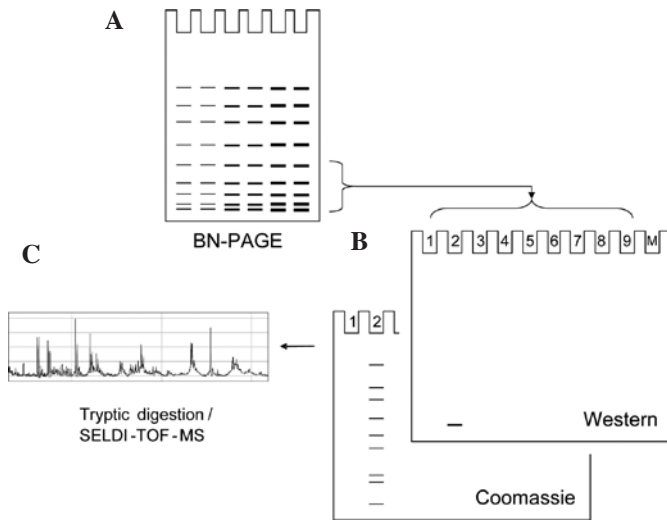


Figure 1. Combination of BN-PAGE and SELDI-TOF-MS. In the BN-PAGE first dimension (A), serum was loaded at different concentrations on a native gel. The gel was stained with Coomassie Brilliant Blue G-250, then stained and separated protein complexes from two equally loaded lanes were transferred to two denaturing second-dimension SDS gels to separate the protein complexes into single proteins. One of these gels was Western blotted with TTR immunodetection to determine which lane contained the TTR complex, and the other was stained with Coomassie Brilliant Blue G-250 (B). Subsequently, the stained bands from the lane containing the TTR complex were excised for tryptic digestion. The digest was analyzed using SELDI-TOF-MS.

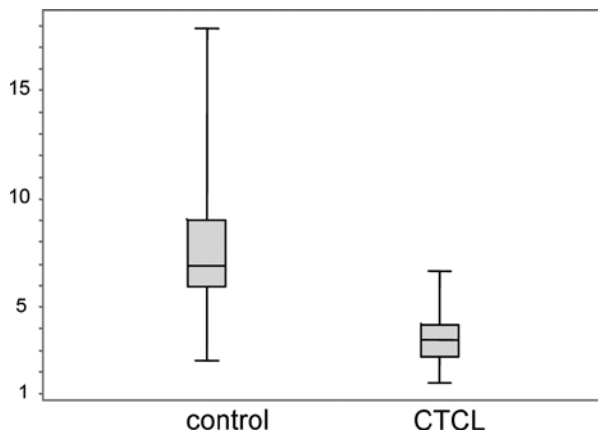


Figure 2. Cluster plotting. The comparison of serum from MF patients and healthy control serum for the 28.1 kDa protein peak resulted in a p-value of 2.87×10^{-6} .

antibody, or IgG as a negative control, were coupled to protein A-agarose. After incubation with serum, the bound proteins were eluted and subjected to SDS-gel separation before Western blotting using a specific anti-apolipoprotein A1 antibody for immunodetection. While a band was visible in the precipitate using the specific antibody for the light chain of the primary antibody, this band was missing in the control using non-specific antibodies (Fig. 3). Thus, a protein-protein interaction between TTR and apolipoprotein A1 was confirmed.

Immunodepletion of differentially expressed proteins. To confirm apolipoprotein A1 as a biomarker in the serum of patients with MF, an immunodepletion study was conducted as follows: anti-apolipoprotein A1 antibody was conjugated

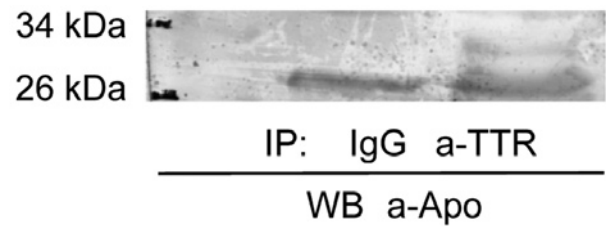


Figure 3. Coimmunoprecipitation of apolipoprotein A1. Specific anti-TTR antibody and non-specific IgG were coupled to protein A-agarose beads and incubated with serum. After washing with CoIP buffer, bound proteins were transferred for SDS-PAGE. CoIP using the TTR antibody revealed a specific band, while the control CoIP using non-specific IgG lacked the signal.

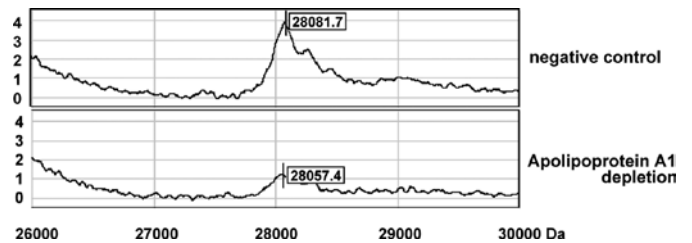


Figure 4. Immunodepletion of apolipoprotein A1. For immunodepletion, a specific anti-apolipoprotein A1 antibody was coupled to protein A-agarose. The beads were incubated with serum and the depleted sample was applied to a copper-activated IMAC 30 ProteinChip array. While the peak with a molecular mass of 28.1 kDa was present in the negative control using a non-specific antibody, the signal from the sample incubated with beads bound to specific anti-apolipoprotein antibody was depleted in this region.

to protein A-agarose. The conjugated antibody was incubated with serum for 45 min at 4°C, and the supernatant was applied to a copper sulfate activated IMAC 30 ProteinChip array. A negative control using non-specific IgG was similarly treated. The protein profiles were analyzed by SELDI-MS. In the experiment using the specific apolipoprotein A1 antibody, the peak with a molecular mass of 28.1 kDa was depleted compared to the negative control using non-specific IgG (Fig. 4).

Discussion

Although treatable in the early stages, most types of cancer become more aggressive as they progress. For CTCL, biomarkers are required to diagnose this disease in the early stages in order to broaden the therapeutic options. Approximately 25% of MF patients with extensive patches or plaques develop progressive disease. To understand the mechanisms of progression, it is not only necessary to identify new biomarkers, but also to obtain information regarding their function, since understanding the proteomic network is a prerequisite for finding new therapies for this disease. Therefore, this study aimed to identify TTR interaction partners, and also to analyze these interaction partners in order to determine whether they themselves serve as biomarkers.

In a previous study, we detected the differential expression of TTR and its modifications in the serum of MF patients and a healthy control group (4), and demonstrated the ability of SELDI-TOF-MS to detect differentially expressed proteins.

Furthermore, we showed that SELDI not only allows for the detection of proteins, but also of their modifications – unlike other techniques, such as 2D-PAGE or ELISA.

The present study is the first to combine the SELDI-TOF technique with BN-PAGE to detect TTR interaction partners and to analyze whether the newly detected interacting proteins can be used as diagnostic biomarkers. Four TTR interaction partners were identified, namely apolipoprotein A1, apolipoprotein A4, RBP-4 and RXR- β . The molecular masses of the interacting proteins were further compared to molecular masses we had previously found for proteins that were differentially expressed between MF patient serum and control serum from an unaffected group of individuals. Apolipoprotein A1 corresponded well to the down-regulated protein (molecular mass 28.1 kDa), as detected by SELDI analysis. An immunodepletion study confirmed that apolipoprotein A1 corresponded to the 28.1-kDa down-regulated protein. In the present study, we describe for the first time the protein interaction of TTR and apolipoprotein A1 in a complex derived from MF serum, and the identification of down-regulated apolipoprotein A1 in MF compared to healthy control serum. While TTR is found down- or up-regulated (4,5,19), apolipoprotein A1 is found down-regulated in other diseases (6,20).

TTR is the major carrier for T₄ and triiodothyronine, which are also present in cerebrospinal fluid (5). The transport of retinol via its interaction with RBPs is also facilitated by TTR. An interaction between TTR and apolipoprotein A1 has also been described by Sousa *et al* (21) in the high-density lipoprotein fraction of plasma. Both proteins are expressed in the liver, and to date no correlation has been found between the down-regulation of these proteins in CTCL or any other diseases.

The most surprising finding of the present study was the presence of RXR- β in the serum of MF patients. Retinoids, such as retinoic acid receptors (RAR) and RXRs, inhibit proliferation and induce differentiation in melanoma cells (22). Both receptor types exert their biological function through three subtypes, α , β and γ . Nuclear retinoid receptors are ligand-dependent transcription factors that bind to cis-acting DNA sequences, known as retinoid acid response elements and retinoid X response elements, which are present in the promoter regions of retinoid-responsive target genes. Abnormalities in the expression and function of RAR and RXR play an important role in the growth of various cancers (23). Retinoids comprise a group of structural and functional analogs of vitamin A. RBP is the specific carrier of retinol in the blood and transports it from the liver to the target tissues. Interaction between RBP and TTR in hepatocytes before their secretion into the bloodstream has already been described by Bellovino *et al* (24). We have demonstrated an interaction between TTR and RBP-4. Moreover, Soares *et al* (25) reported RBP-4 to play a role in Portuguese TTR V30M amyloid polyneuropathy.

RXR is also regulated via a pharmacological response to bexarotene. The RXR specific retinoid bexarotene is used in the treatment of CTCL (26). Therefore, changes in the serum level of TTR and its interaction partners, which are responsible for retinoid binding to its RAR or RXR receptors, may have consequences for cell proliferation.

In conclusion, the combination of BN-PAGE and SELDI-TOF-MS allows the detection of endogenously expressed protein interaction partners. In the case of RBP-4 and RXR, this indicates a connection between the common tumor marker TTR and tumor progression in CTCL.

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