Paraproteins of familial MGUS/multiple myeloma target family-typical antigens: hyperphosphorylation of autoantigens is a consistent finding in familial and sporadic MGUS/MM

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Abstract

Paratarg-7 (P-7) is a frequent paraprotein target in MGUS, multiple myeloma (MM) and Waldenström’s macroglobulinemia. Patients with paratarg-7-specific paraproteins carry a hyperphosphorylated paratarg-7 (pP-7). Because pP-7 carrier state is dominantly inherited, we determined the paraprotein targets in 4 families with familial MGUS/MM. No antigenic target was identified for the paraproteins from 2 members of one family. Paraproteins from affected members of two other families targeted paratarg-7, and paraproteins from 4 affected members of a fourth family targeted paratarg-8, which is encoded by the ATG13 gene. Paratarg-8 was hyperphosphorylated in the affected family members (pP-8) and pP-8 carrier state is inherited in a dominant fashion. Six additional autoantigenic non-familial paraprotein targets were also hyperphosphorylated in the respective patients compared to normal controls. We conclude that paraproteins of affected members with familial MGUS/MM share family-typical hyperphosphorylated antigens and hyperphosphorylation of paraprotein targets might be a general mechanism underlying the pathogenesis of MGUS/MM.

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Introduction

Using a human fetal brain-derived protein macroarray in a modified SEREX approach we identified paratarg-7 as the target of 15% of IgA or IgG paraproteins and 11% of IgM paraproteins. All patients with paratarg-7 specific paraproteins are carriers of a hyperphosphorylated version of the protein (pP-7) and this hyperphosphorylation is inherited in a dominant fashion. pP-7 carrier state is associated with an increased risk to develop IgA/IgG-MGUS/MM (odds ratio: 7.9) and IgM-MGUS (odds ratio 6.5). Since pP-7 is the first molecularly defined risk factor for any hematological neoplasm known to date, we set out to define the antigenic targets of paraproteins from affected members of families with familial MGUS/MM.

Methods

Patients and controls

This study was approved by the local ethical review board ("Ethikkommission der Ärztekammer des Saarlandes") and conducted according to the Declaration of Helsinki. Peripheral blood and serum samples from 31 healthy and 10 affected members of 4 families with familial MGUS/MM were collected after obtaining written informed consent.

Serum screening on high-density protein arrays, immunoblot analyses, absorption studies, isoelectric focusing (IEF), and phosphatase treatment of paraprotein targets

These were performed as described before. Absorption studies using His6-tagged paratarg-8 protein or erythrocyte lysates were performed as described before for paratarg-7. Similarly, Western Blot analyses, isoelectric focusing and phosphatase treatment of all paraprotein targets were performed as described before for paratarg-7.
Paratarg-7 and Paratarg-8 ELISA

The ELISAs using full-length recombinant paratarg-7 was performed as described by us previously. For the paratarg-8 ELISA recombinant paratarg-8 was used as a coat, and the ELISA were performed according to standard protocols using the paraproteins at a dilution of 1:10^6.

Results / Discussion

Four families with ≥2 cases of MGUS/MM were included in this study. No antigenic target was identified for the paraproteins from a brother and a sister of the first family, both affected by MM. Two sisters diagnosed with MGUS in the second family whose pedigree had been described before and two siblings with MM in the third family (S Fig. 1) all had an anti-paratarg-7 specific paraprotein and were carriers of pP-7.

Identification and characterization of paratarg-8

The paraproteins from 2 MGUS and 2 MM patients of the fourth family (Fig. 1) did not react with paratarg-7. Screening of the protein macroarray identified a single protein as the antigenic target of all 4 paraproteins from these family members affected by MGUS/MM. Sequence analysis showed that this protein was coded by the ATG13 gene, a member of the „autophagy regulatory complex“ family of genes. The specificity and paraprotein-mediated nature of the observed anti-paratarg-8 reaction was shown by the high titer of the reaction (1:10^8) and by absorption with recombinant paratarg-8 (S Fig. 2).

To investigate the prevalence of paraproteins with paratarg-8 specificity, 300 paraprotein-containing sera were tested for reactivity with paratarg-8 by ELISA. Of these, only one reacted with paratarg-8 (titer 1:10^7). Sequence analysis of paratarg-8 derived from patients with a
paraprotein-8 specific paraprotein and healthy controls excluded mutations or polymorphisms as a cause of the autoimmunogenicity of paratarg-8. All lysates from patients with a paraprotein with anti-paratarg-8 or non-paratarg-8 specificity and healthy controls showed identical bands in the Western blots (data not shown). However, erythrocyte lysates from healthy controls and from patients with a paratarg-8 specific paraprotein migrated differently in the IEF (S Fig. 3). After phosphatase treatment all lysates showed identical bands in the IEF, regardless of whether they were derived from healthy controls, from patients having a paraprotein with specificity for paratarg-8, or from patients with a paraprotein that did not bind to paratarg-8. Differential banding in the IEF before and after treatment with alkaline phosphatase demonstrated that paratarg-8 is phosphorylated in healthy individuals, though to a lesser degree than in MGUS/MM patients with an anti-paratarg-8 reactive paraprotein. All 4 MGUS/MM patients with an anti-paratarg-8 paraprotein expressed hyperphosphorylated paratarg-8, but hyperphosphorylation of paratarg-8 was not observed in MM/MGUS patients whose paraprotein did not bind to paratarg-8.

Only 1/200 healthy Caucasians was shown to be carrier of pP-8 as shown by IEF, demonstrating that pP-8 has a much lower prevalence than pP-7 carrier state.

**Phosphorylation state of other autoantigenic targets of paraproteins**

All other molecularly defined autoantigenic targets of paraproteins from sporadic, non-familial cases of MGUS/MM identified by our group to date, where cells from the respective patients were available, were tested by IEF before and after phosphatase treatment (Tab. 1). In all these cases the patients’ paraprotein target was hyperphosphorylated compared to normal controls (S Fig. 4).
Paraproteins from patients of families with multiple cases of MGUS/MM are directed against the same autoantigen: while no target could be identified for the paraproteins from 2 siblings of family #1, the paraproteins from each 2 affected family members of family #2 and family #3 (S Fig. 1), respectively, recognized paratarg-7, and the paraproteins from family #4 (Fig. 1) reacted with paratarg-8. The second intriguing finding of our study is that the autoantigens detected by the autologous paraproteins were hyperphosphorylated in the respective patients compared to normal controls in all (8/8) cases where this analysis was possible. The fact that all autoantigenic targets (and possible stimuli) of paraproteins molecularly defined to date are hyperphosphorylated suggests that hyperphosphorylation might be a general mechanism underlying the pathogenesis of MGUS/MM that is operative in many more cases than those with the 8 autoantigenic paraprotein targets described in this study. The elucidation of how this mechanism induces the clonal evolution of a B-cell clone with specificity for the respective autoantigen as well as the question why some carriers of hyperphosphorlyated antigens develop MGUS/MM while others do not, can now be addressed using specific molecular tools.

There have been a number of studies of familial multiple myeloma, implicating both environmental and inherited factors\(^7-11\), but results have been inconsistent\(^{12}\). pP-7 and pP-8 are the first molecularly characterized structures that provide a plausible explanation for the familial clustering of cases of MGUS/MM, at least in cases with a paratarg-7 (2 families) or paratarg-8 (one family to date) specific paraprotein. While a molecular mimicry between these autoantigens and infectious agents can not be definitely excluded, this mechanisms is highly unlikely for two reasons: first, a data bank search comparing the autoantigenic targets with sequences from bacteria or viruses did not reveal significant homologies (data not shown); 2\(^{nd}\), it would not explain why only the hyperphosphorylated, but not the wildtype protein acts as an autoantigen. The hyperphosphorylation of the autoantigens appears to be the most obvious likely reason for
their autoimmunogenicity. Indeed, „phosphoepitopes“ were reported to induce stronger CD8$^{+}$ and CD4$^{+}$ T-cell responses than their non-phosphorylated counterparts. Whether the hyperphosphorylated autoantigenic targets of paraproteins induce the development of MGUS/MM by chronic antigenic stimulation or whether they are only a marker or an epiphenomenon of another susceptibility to develop MGUS/MM can now be investigated in the respective patients and their (not yet) affected relatives. Logical next steps are the identification of the kinases and phosphatases responsible for maintenance of the hyperphosphorylated state and the identification of the mutation or polymorphism(s) underlying the posttranslational modification of the autoantigenic targets of paraproteins.

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**Author contributions**

SG designed the experiments and wrote the manuscript; K-D P designed the experiments; NF and ER performed and analysed the experiments. FZ and LT recruited and helped analyze the family investigation for the study. DW, VW and JL recruited and analyzed the American family for the study. HL and MP designed the study and wrote the manuscript.
Financial disclosures

KDP and MP have applied for a relevant patent. Otherwise, none of the authors has a conflict of interest.

References


Figure Legends

Figure 1. Pedigree of members of family #4 including 2 patients with MM and 2 with MGUS with a paratarg-8 specific paraprotein carrying the hyperphosphorylated paratarg-8. A: The pedigree shows the family (family # 4) of two patients with MM (III.1 and IV.1) and two patients with MGUS (IV.3 and IV.5), all having paratarag-8 reactive paraproteins and carrying the hyperphosphorylated state of this protein. The pedigree is a part of a pedigree previously published 7. B: Immunostaining of lysate bands derived from whole peripheral blood lysates from family members carrying wild-type (III.2; IV.2; IV.4; V.1) and hyperphosphorylated paratarg-8 (III.1; IV.1; IV.6; V.2) after IEF. The numbers indicate family members in different generations.
Table 1: Hyperphosphorylation of autoantigenic paraprotein targets

<table>
<thead>
<tr>
<th>Paratarg #</th>
<th>Antigen</th>
<th>type of antigen</th>
<th># binding</th>
<th>subtype***</th>
<th>Phosphorylation state</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cylicin 2</td>
<td>allaontigen*</td>
<td>1/115 non-familial cases</td>
<td>1 x IgA-λ</td>
<td>no data available</td>
</tr>
<tr>
<td>2</td>
<td>TPP2</td>
<td>autoantigen**</td>
<td>1/115 non-familial cases</td>
<td>1 x IgG-k</td>
<td>hyperphosphorylated</td>
</tr>
<tr>
<td>3</td>
<td>IGFBP-2</td>
<td>autoantigen**</td>
<td>1/115 non-familial cases</td>
<td>1 x IgG-k</td>
<td>no data available</td>
</tr>
<tr>
<td>4</td>
<td>Porcine kinesin</td>
<td>heteroantigen</td>
<td>1/115 non-familial cases</td>
<td>1 x IgG-k</td>
<td>no data available</td>
</tr>
<tr>
<td>5</td>
<td>Microtubuli-assoc. protein</td>
<td>autoantigen**</td>
<td>2/103 non- familial cases</td>
<td>1 x IgM-κ, 1 x IgG-λ</td>
<td>hyperphosphorylated</td>
</tr>
<tr>
<td>6</td>
<td>LAPTM5</td>
<td>autoantigen**</td>
<td>3/103 non- familial cases</td>
<td>2 x IgG-λ, 1 x IgG-k</td>
<td>hyperphosphorylated</td>
</tr>
<tr>
<td>7</td>
<td>SLP-2</td>
<td>autoantigen**</td>
<td>4 cases from 2 families; 76 / 650 non-familial cases</td>
<td>see Ref. 2</td>
<td>hyperphosphorylated</td>
</tr>
<tr>
<td>8</td>
<td>ATG13</td>
<td>autoantigen**</td>
<td>4 cases from 1 family; 1 / 300 non-familial cases</td>
<td>3 x IgG-λ, 1 x IgG-k</td>
<td>hyperphosphorylated</td>
</tr>
<tr>
<td>9</td>
<td>RSP16</td>
<td>autoantigen**</td>
<td>1/300 non-familial cases</td>
<td>1 x IgG-k</td>
<td>hyperphosphorylated</td>
</tr>
<tr>
<td>10</td>
<td>SPAG7</td>
<td>autoantigen**</td>
<td>2/300 non-familial cases</td>
<td>2 x IgG-λ</td>
<td>hyperphosphorylated</td>
</tr>
<tr>
<td>11</td>
<td>SIVA</td>
<td>autoantigen**</td>
<td>2/300 non-familial cases</td>
<td>1 x IgG-κ, 1 x IgA-λ</td>
<td>hyperphosphorylated</td>
</tr>
</tbody>
</table>

* Cylicin 2 is sperm-specific, hence it functioned as an alloantigen in the respective female patient

** autoantigen, since expressed in the autologous patient’s tissues

*** refers to the number of paraproteins with the respective specificity among all paraproteins tested for this specificity
Figure 1
Paraproteins of familial MGUS/multiple myeloma target family-typical antigens: hyperphosphorylation of autoantigens is a consistent finding in familial and sporadic MGUS/MM

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