TRPM1 Autoantibodies in Melanoma Patients Without Self-Reported Visual Symptoms

Robert M. Duvoisin,1 Gaoying Ren,1 Tammie L. Haley,1 Matthew H. Taylor,2,3 and Catherine W. Morgans1

1Physiology and Pharmacology, Oregon Health and Science University, Portland, Oregon, United States
2Hematology and Oncology, Oregon Health and Science University, Portland, Oregon, United States
3Knight Cancer Institute, Oregon Health and Science University, Portland, Oregon, United States

CORRESPONDENCE: Catherine W. Morgans, Department of Physiology and Pharmacology, L-354, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239, USA; morgansc@ohsu.edu.

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PURPOSE. Melanoma-associated retinopathy (MAR) is a paraneoplastic syndrome associated with cutaneous malignant melanoma (CMM). Visual symptoms include night blindness, photopsia, and reduced-contrast sensitivity. An abnormal ERG b-wave and the presence of anti-bipolar cell autoantibodies, including autoantibodies reacting with the ON-bipolar cell TRPM1 channel, help to confirm the diagnosis. The goal of this study was to determine if CMM patients without visual symptoms also express anti-TRPM1 autoantibodies.

METHODS. Serum samples from 15 CMM patients were tested using three assays: immunofluorescent labeling of TRPM1-transfected HEK cells, immunofluorescent labeling of retinal sections from wild-type and TRPM1 knockout mice, and immunoblot detection of a bacterially produced recombinant TRPM1 peptide.

RESULTS. Serum specimens from 5 of the 15 CMM patients without declared visual symptoms were positive for anti-TRPM1 autoantibodies in at least one of the three assays. One of 50 control sera from patients not known to have cancer was also weakly reactive with the TRPM1 peptide.

CONCLUSIONS. Autoantibodies against TRPM1 are present in CMM patient sera without self-reported visual symptoms. Most patients had advanced (stage III and IV) disease and were undergoing aggressive treatments, including immunotherapy. It is unknown if immunotherapy affects the expression of TRPM1 autoantibodies. The presence of TRPM1 autoantibodies may predispose patients for MAR.

Keywords: retina, autoimmune response/disease, MAR, bipolar cells, TRPM1
TRPM1 Autoantibodies in Cutaneous Melanoma

Animals

Adult wild-type and TRPM1 knockout mice of both sexes were used in this study. All mice were maintained on a 12-hour light-dark cycle, provided food and water ad libitum, and used for experiments in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Retina transversal sections were used for screening of CMM serum immunoreactivity. All animal procedures were approved by the OHSU Institutional Animal Care and Use Committee.

Cell Culture, Transfection, and Immunocytochemistry

HEK293 cells, seeded onto poly-lysine coated coverslips, were transfected with plasmids derived from pEGFP-C3 (Clontech, Mountain View, CA, USA) encoding human TRPM1 fused at the C-terminus of enhanced green fluorescent protein (EGFP), using Effectene (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Twenty-four to 72 hours after transfection, cells were fixed for 1 minute by immersion in cold 4% paraformaldehyde and then processed for immunofluorescence according to the protocol previously described. Using dilutions of patient serum (1:100 to 1:1000), and anti-human immunoglobulin (IgG) conjugated to Alexa Fluor 594 (1:1000; Invitrogen, Carlsbad, CA, USA). Fluorescence images were acquired with an Olympus (Tokyo, Japan) Fluoview FV1000 confocal microscope using a ×60/1.42 oil immersion objective. Image brightness and contrast were enhanced using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA, USA).

For retinal immunofluorescence, freshly dissected mouse eyes were hemisected and the front of the eye and lens were discarded. The remaining eyecup containing the retina was fixed by immersion in ice-cold 4% paraformaldehyde for 20 minutes, washed in ice-cold PBS, then cryoprotection by consecutive incubations in cold 10%, 20%, and 30% sucrose; then stored at −80°C until use. Sections were thawed and processed for immunofluorescence confocal microscopy as described above for HEK293 cells and previously.

TRPM1 Peptide Production

A peptide derived from mouse TRPM1 (amino acids 284–430) corresponding to the MAR epitope was produced in bacterial culture. This sequence is 91% identical between mice and humans. For this, the pEGFP-C3-Pst-Acc plasmid was digested with EcoRI and BamHI and the TRPM1-encoding fragment was subcloned into the pET28a vector (Novagen; EMD-Millipore Sigma, Burlington, MA, USA) between the Nhel and BamHI sites. A 100-ml culture was inoculated with 1 ml from an overnight culture, grown at 37°C until OD550 = 0.5, and protein expression was induced by addition of Isopropyl β-D-1-thiogalactopyranoside for 1 hour. Cells were pelleted, resuspended in BugBuster Master Mix (Novagen; EMD-Millipore Sigma), and incubated for 20 minutes. Inclusion bodies were then pelleted and washed in BugBuster twice.

Western Blot Analysis

Untransfected HEK293 cells were collected in RIPA buffer, with protease inhibitor cocktail (Cell Signaling Technology, Danvers, MA, USA) for Western blot analysis. Half of the HEK293 cell lysate was spiked with the TRPM1 peptide at a concentration of 1 ng/μL. Lysates with and without TRPM1 peptide were electrophoresed on precast 4% to 12% polyacrylamide gradient gels (Novex; Invitrogen). The separated proteins were electrophoretically transferred to polyvinylidene difluoride membranes, which were then probed with human CMM patient sera (1:100 to 1:1000) followed by anti-human IgG IRDye 680CW (1:10,000). Blots were visualized with an Odyssey infrared imaging system (LI-COR, Lincoln, NE, USA).

RESULTS

TRPM1 Autoantibodies in CMM Patient Sera

Serum samples from 14 patients with stage III and IV CMM and one stage I patient, none of whom reported visual symptoms, were tested for TRPM1 autoantibodies by three methods (Table). The samples were first tested by immunofluorescent labeling of HEK293 cells transfected to express a polypeptide corresponding to the N-terminal, cytoplasmic domain of human TRPM1 fused to the C-terminus of green fluorescent protein (GFP–TRPM1) using methods reported previously for screening MAR patient sera. Transfected cells were identified by GFP fluorescence, and represented between 10% and 50% of the cells. Serum samples that displayed greater immunofluorescence (Alexa Fluor 594 signal) on GFP-positive cells compared with neighboring, untransfected cells were scored as positive for TRPM1 autoantibodies. By this assay, 2/15 CMM sera (CMM01 and CMM14) were found to be immunoreactive with GFP–TRPM1 (Fig. 1A, Table). Of 50 normal sera (from persons with no known history of cancer), none were found to label GFP–TRPM1–transfected HEK293 cells (not shown).

The CMM serum samples were also tested for TRPM1 autoantibodies by immunofluorescent labeling of wild-type and TRPM1 knockout mouse retina sections, as previously described for MAR sera. This assay is likely to be less sensitive than the labeling of transfected cells due to the lower concentration of TRPM1 in retinal bipolar cells compared with the transfected cells. Nevertheless, the two patient samples that immunolabeled GFP–TRPM1 transfected HEK293 cells (CMM01 and CMM14), also selectively labeled retinal bipolar neurons in wild-type, but not TRPM1 knockout mouse retina sections (Fig. 1B). None of the 50 control sera reacted with bipolar cells on mouse retina sections (not shown).

TRPM1 Autoantibodies in CMM Target an Epitope in the N-terminal, Cytoplasmic Domain

TRPM1 is a large protein composed of six transmembrane segments, and N- and C-terminal cytoplasmic domains, yet epitope mapping indicates that TRPM1-immunoreactive MAR sera react with a discrete region within the N-terminal cytoplasmic domain of TRPM1 (amino acids 284–430). Therefore, we tested CMM patient sera for immunoreactivity against a bacterially expressed, recombinant peptide encompassing this sequence. To control for nonspecific antibody binding, the peptide was added to an untransfected HEK293 cell lysate, and alternating lanes of HEK293 lysate and minus the TRPM1 peptide were separated by SDS-PAGE and immunoblotted with dilutions of CMM patient sera. Serum samples that labeled the peptide band more intensely than the endogenous HEK293 bands were scored positive for immunoreactivity with the TRPM1 peptide. MAR patient sera strongly labels the TRPM1 peptide (Fig. 2). The two CMM serum samples that labeled mouse retina sections and TRPM1–transfected HEK293 cells (Fig. 1), also reacted with the TRPM1 peptide on immunoblots (Fig. 2, Table). Three additional CMM sera (CMM03, -13, -15) were also positive on Western blots of the TRPM1 peptide, despite failing to label GFP–TRPM1–transfected cells or retina sections, consistent with the peptide
<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex-Age</th>
<th>Stage</th>
<th>HEK293</th>
<th>Retina</th>
<th>Peptide</th>
<th>Treatment/Status</th>
<th>Blood Collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAR</td>
<td>IV</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>MAR patient 2 in Xiong et al., 2013. Treatment and status unknown.</td>
<td>Blood collected after MAR diagnosis.</td>
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<tr>
<td>CMM01 F-23</td>
<td>IV</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Dabrafenib/trametinib, no response; high dose IL-2: no response; ipilimumab: no response; pembrolizumab: stable disease 1 year. Deceased.</td>
<td>Advanced metastatic disease at the time of blood collection.</td>
</tr>
<tr>
<td>CMM03 M-35</td>
<td>IV</td>
<td></td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>Vemurafenib: complete response (over 6 years and ongoing). No radiographic evidence of disease.</td>
<td>Blood collected after complete response to therapy.</td>
</tr>
<tr>
<td>CMM04 M-46</td>
<td>IV</td>
<td></td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>High dose IL-2: no response; ipilimumab: no response; pembrolizumab: partial response (4 years and ongoing).</td>
<td>Advanced metastatic disease at the time of blood collection.</td>
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<tr>
<td>CMM05 F-69</td>
<td>IV</td>
<td></td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>Dabrafenib/trametinib: partial response (5 years and ongoing).</td>
<td>Low-volume metastatic disease at the time of blood collection.</td>
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<tr>
<td>CMM06 M-72</td>
<td>IV</td>
<td></td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>Vemurafenib: responded for 5 months; ipilimumab: no response; pembrolizumab: no response. Deceased.</td>
<td>Advanced metastatic disease at the time of blood collection.</td>
</tr>
<tr>
<td>CMM08 M-43</td>
<td>IV</td>
<td></td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>Clinical trial with an investigational MEK inhibitor: stable disease for 14 months; ipilimumab: no response; clinical trial with investigational anti-PD-L1 antibody: partial response (4 years and ongoing).</td>
<td>Advanced metastatic disease at the time of blood collection.</td>
</tr>
<tr>
<td>CMM09 M-81</td>
<td>IV</td>
<td></td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>Ipilimumab: complete response still ongoing (5 years). No radiographic evidence of disease.</td>
<td>Blood collected after complete response to therapy.</td>
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<tr>
<td>CMM10 M-77</td>
<td>IV</td>
<td></td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>Clinical trial with investigational BRAF inhibitor: partial response lasting 18 months; nivolumab: partial response (3.5 years and ongoing).</td>
<td>Advanced metastatic disease at the time of blood collection.</td>
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<tr>
<td>CMM11 F-54</td>
<td>IV</td>
<td></td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>High dose IL-2: no response; ipilimumab: no response; pembrolizumab: partial response (4 years and ongoing).</td>
<td>Advanced metastatic disease at the time of blood collection.</td>
</tr>
<tr>
<td>CMM13 ÷53</td>
<td>IV</td>
<td></td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>Dabrafenib/trametinib: complete response (ongoing 4 years).</td>
<td>Blood collected after complete response to therapy.</td>
</tr>
<tr>
<td>CMM15 ÷64</td>
<td>I</td>
<td></td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>Complete excision.</td>
<td>Blood collected 14 years after surgery.</td>
</tr>
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immunoblot being a more sensitive assay. Of the 50 normal serum samples, one displayed weak reactivity with the peptide (N1 in Fig. 2).

**DISCUSSION**

Our results demonstrate that the incidence of TRPM1 autoantibodies is more widespread in melanoma patients than is suggested by the incidence of clinically diagnosed MAR. Combining the results of the three assays, TRPM1 autoantibodies were detected in 4 of 14 stage III and IV CMM patients and one stage I patient without reported visual symptoms (Table). Despite having detectable levels of TRPM1 autoantibodies in their blood, none of the CMM patients in this study had been diagnosed with MAR. Three of these patients were under treatment when their blood draws were taken and none had complained of adverse changes in their vision. There are several reasons why CMM patients with TRPM1 autoantibodies may not report vision changes. In some cases, the autoantibody titer may be too low to affect retinal function to an extent that will be noticeable to the patient; alternatively, the autoantibody

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**Figure 1.** CMM patient sera react with TRPM1 in transfected cells and label retinal bipolar cells. (A) HEK293 cells transiently transfected with the N-terminal, cytoplasmic domain of human TRPM1 fused to GFP were labeled by immunofluorescence with CMM patient sera. The GFP fluorescence identifies the transfected cells, and untransfected cells serve as a control for background immunofluorescence. The left panels show immunoreactivity with CMM patient sera; the GFP images (middle) show cells transfected with TRPM1-GFP; DIC images (right) show all cells (transfected and untransfected). The scale bar represents 20 μm. (B) Serum samples CMM01 and CMM14 label retinal bipolar cells by immunofluorescence on mouse retinal cryosections. Scale bar represents 10 μm.

**Figure 2.** TRPM1 autoantibodies from CMM patients bind to a discrete region within the N-terminal cytoplasmic domain of TRPM1. HEK293 cell extracts with (+) and without (−) recombinant TRPM1 peptide were Western blotted with serum from a melanoma patient diagnosed with MAR, sera from five melanoma patients without a MAR diagnosis (CMM01, -03, -13, -14, -15), and sera from six patients with no known cancer (N1-N6).
TRPM1 is known to be downregulated in CMM, yet the expression of TRPM1 autoantibodies in the CMM patients in this study is considerably lower than in patients diagnosed with MAR (see Fig. 2). All prior reports of TRPM1 autoantibodies in the literature are correlated with MAR or paraneoplastic retinopathy, thus the detection of TRPM1 autoantibodies in melanoma patients without reported visual symptoms suggests that these patients are at risk of developing MAR.

It is currently unknown why TRPM1 becomes immunogenic in CMM. This is especially puzzling because expression of TRPM1 is known to be downregulated in CMM, yet the autoantibodies are typically detected in advanced stage patients. One possible explanation is that the TRPM1 mRNA is abnormally spliced in metastatic melanoma cells leading to the formation of truncated TRPM1 polypeptides in melanocytes, but that several short transcripts encoding N-terminal polypeptides appear upregulated. Indeed, melanoma-specific, truncated TRPM1 N-terminal polypeptides have been reported.

Ten of the 13 stage IV patients in this study had been treated with immune checkpoint inhibitors (ICIs), and one of the serum samples from these patients (CMM01) was positive for TRPM1 autoantibodies. It is presently unknown whether ICIs influence the production of TRPM1 autoantibodies, but ICIs are generally associated with improved immune-related adverse events, and ICI treatment has been found to correlate with an increase in autoantibodies against NMDA receptors in melanoma patients.

It is becoming increasingly evident that the rate of cancer progression is dramatically influenced by the patient’s immune system, and the development of spontaneous immunity is generally a good prognostic indicator for CMM patients. Indeed, the occurrence of MAR is correlated with longer survival. Consistent with this correlation, four of the five TRPM1-reactive CMM serum samples in this study were from patients who were in remission (no evidence of disease). It remains to be determined whether TRPM1 autoantibodies may be a positive prognostic indicator.

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