Gene Expression Profile of Inflammatory Myopathy with Malignancy is Similar to that of Dermatomyositis rather than Polymyositis

Tomoko Noda, Masahiro Iijima, Seiya Noda, Shinya Maeshima, Hirotaka Nakanishi, Seigo Kimura, Haruki Koike, Shinsuke Ishigaki, Yohei Iguchi, Masahisa Katsuno and Gen Sobue

Abstract

Objective  An association has been reported between inflammatory myopathies (IMs), which include polymyositis (PM) and dermatomyositis (DM), and malignancy, and the concept of cancer-associated myositis (CAM) was recently proposed. We herein attempted to determine the features and etiologies of these myopathies.

Methods  We analyzed the gene expression levels via microarray and real-time quantitative reverse transcription polymerase chain reaction analyses to identify genes that were specifically upregulated or downregulated with suspected inflammatory involvement and verified the microarray data via an immunohistochemical (IHC) analysis in additional cases.

Patients  We selected 14 patients with the following conditions: PM without malignancy (n=3), DM without malignancy (n=3), CAM (n=3), and Controls (no pathological changes or malignancy; n=5).

Results  PM was distinct from DM and CAM in a clustering analysis and exhibited the highest numbers of overexpressed genes and specific pathologies in a gene ontology analysis. The IHC analysis confirmed the gene expression results.

Conclusion  PM is associated with severe inflammatory pathological findings, primarily in the cell-mediated immune system. DM and CAM exhibit similarities in the gene expression and IHC results, which suggest that humoral immunity is the main etiology for both myopathies, indicating the importance of cancer screening in patients with IMs, particularly DM.

Key words: polymyositis, dermatomyositis, cancer-associated myositis, gene expression, immunohistochemistry


Introduction

Inflammatory myopathies (IMs), which include polymyositis (PM) and dermatomyositis (DM), comprise a heterogeneous group of disorders characterized by muscle inflammation and weakness (1-4). The criteria developed by Bohan and Peter emphasize the clinical features of these disorders, particularly the presence or absence of a rash (1, 2). However, many other studies have revealed the etiological differences between DM and PM. According to the criteria published by Dalakas and Hohlfeld, specific pathological findings are required for a definitive diagnosis (5). Accordingly, various criteria, each with a particular specificity and sensitivity, have been proposed to classify PM and DM (6). Recently, immune-mediated necrotizing myopathy and sporadic body myositis have been recognized as IMs, and the relationship between many autoantibodies and pathological...
findings has attracted increasing attention in study of IMs (7).

Specifically, PM is presumed to involve an antigen-specific, cell-mediated immune response, whereas DM is thought to involve an autoimmune, humoral immune-mediated microangiopathy (8), although the antigens and triggers of these autoimmune responses remain unknown.

The association between IMs, particularly DM, and malignancy has been well described (9-14). According to the first meta-analysis conducted to preliminarily confirm this association, malignancy was found to occur in 25% and 10-15% of adult patients with DM and PM, respectively, within 0-5 years of disease onset (15).

In a recent meta-analysis study, the pooled risk ratios or standardized incidence ratios for patients with PM, DM, and PM/DM relative to the general population were 1.62 (95% confidence interval: 1.19-2.04), 5.50 (4.31-6.70), and 4.07 (3.02-5.12), respectively (16). Additionally, many reports have described cancer-associated myositis (CAM) (17-20). This disorder is thought to arise in response to the common expression of autoantigens on both cancer and muscle tissues, however, the etiological mechanism remains unclear (21, 22). Because the pathology of CAM has not been established and many cases of IM do not fulfill the criteria described by Dalakas and Hohlfeld, it is difficult to diagnose PM, DM, and CAM from pathological muscle findings alone.

Microarray techniques can be used to define clinically significant disease subtypes via molecular profiling of a tissue from a quantitative list of relative gene expression levels. This profile may serve as a “signature” of the large-scale gene expression. In this study, we used microarray techniques and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) to analyze the characteristics of PM, DM, and CAM and confirmed our findings via immunohistochemical (IHC) analyses.

Materials and Methods

Patients

This retrospective study was approved by the ethical committee of Nagoya University. We retrospectively evaluated patients who had undergone a muscle biopsy. The enrolled patients were divided into four groups according to their condition: PM, DM, CAM, and Controls. All patients with IMs fulfilled the Bohan and Peter criteria for definite or probable IMs. Patients with PM and DM had no history of malignancy before the muscle biopsy and passed an interval of >3 years after biopsy without a diagnosis of malignancy. In patients with CAM, malignancy was diagnosed concurrently with the onset of myopathy. None of the patients had received any treatment, including immunotherapy, prior to the muscle biopsy. The Control patients had undergone a muscle biopsy due to suspicion of a myopathy. However, they did not present with high plasma creatine kinase levels or any inflammatory changes evident in their muscle biopsies.

Analytical approach

We initially constructed gene expression profiles using microarray techniques and analyzed the gene ontology (GO) of each profile. According to our gene expression analysis, we verified the genes with expected inflammatory and pathological involvement using real-time qRT-PCR and IHC techniques. Table 1 presents the clinical data of 14 patients (PM: 3 cases, DM: 3 cases, CAM: 3 cases, and Control: 5 cases) whose specimens were subjected to microarray and real-time qRT-PCR analyses.

In the microarray analysis, the enrolled PM and DM patients fulfilled both the Bohan and Peter criteria and the Dalakas and Hohlfeld criteria. The criteria developed by Bohan and Peter for IMs were found to exhibit an extremely poor specificity (29%), whereas the criteria published by Dalakas and Hohlfeld in 2003 exhibited a high specificity (99%) and sensitivity (77%) (23). Accordingly, we used the latter criteria to identify patients for our microarray analysis and assumed that the selected patients in both the PM and DM groups were relatively homogeneous.

Namely, patients 1-3 in the PM group had endomyosial CD8-positive cytotoxic T cell inflammation. Patients 4-6 in the DM group had “skin rash” clinically and perifascicular atrophy pathologically. Patient 4 became aware of mild dysphasia at an earlier stage of the disease, but rash and weakness were observed following dysphasia. Therefore, we considered that patient 4 had DM and patients 4-6 comprised a homogeneous DM group. Not all cases showed typical PM and DM pathology in the CAM group, however, we could observe the infiltration of inflammatory cells. Anti-Jo-1 antibody was positive in patient 5 (DM), but aminoacyl tRNA synthetase (ARS) antibodies and other autoantibodies were negative in the other patients (patients 1-4, 6-9).

We enrolled additional patients for the IHC analysis to verify the results of microarray and real-time qRT-PCR analyses. The patients enrolled for the IHC analysis were as follows: 14 patients with PM (4 men, 10 women; mean age, 63 years), 13 patients with DM (4 men, 9 women; mean age, 53 years), 25 patients with CAM (13 men, 12 women; mean age, 60 years), and 11 Control patients (7 men, 4 women; mean age, 43 years).

In the CAM group, the following malignancies were observed: lung cancer (n=6), gastric cancer (n=5), breast cancer (n=3), malignant lymphoma (n=3), tongue cancer (n=1), laryngeal cancer (n=1), esophageal cancer (n=1), hepatocellular carcinoma (n=1), bile duct cancer (n=1), uterine corpus cancer (n=1), ovarian cancer (n=1), and prostate cancer (n=1).

Gene expression analysis

RNA extraction

Frozen muscle tissue samples (50-100 mg) were homogenized in 1 mL of ISOGEN (Nippon Gene, Tokyo, Japan).
Table 1. Clinical Features of Patients in the Microarray and Real-time qRT-PCR Study.

<table>
<thead>
<tr>
<th>Pt No.</th>
<th>Age (years) /sex</th>
<th>Diagnosis</th>
<th>Disease Duration</th>
<th>Initial symptom</th>
<th>Rash</th>
<th>CK (IU/L)</th>
<th>Muscle biopsied/MRC Grade</th>
<th>Treatment for myopathy</th>
<th>Malignancy pathology/metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>62/M</td>
<td>PM</td>
<td>6M</td>
<td>Weakness</td>
<td>-</td>
<td>3,329</td>
<td>Quadriceps/3</td>
<td>PSL</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>77/F</td>
<td>PM</td>
<td>6M</td>
<td>Weakness</td>
<td>-</td>
<td>312</td>
<td>Biceps/4</td>
<td>PSL + CyA</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>65/F</td>
<td>PM</td>
<td>3M</td>
<td>Weakness</td>
<td>-</td>
<td>10,931</td>
<td>Biceps/4</td>
<td>PSL</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>62/F</td>
<td>DM</td>
<td>24M</td>
<td>Dysphasia</td>
<td>+</td>
<td>232</td>
<td>Biceps/4</td>
<td>PSL</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>35/M</td>
<td>DM</td>
<td>6M</td>
<td>Rash</td>
<td>+</td>
<td>863</td>
<td>Biceps/4</td>
<td>PSL</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>60/M</td>
<td>DM</td>
<td>5M</td>
<td>Rash</td>
<td>+</td>
<td>307</td>
<td>Biceps/4</td>
<td>PSL</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>59/M</td>
<td>CAM</td>
<td>1M</td>
<td>Rash</td>
<td>+</td>
<td>1,101</td>
<td>Quadriceps/4</td>
<td>PSL</td>
<td>Larynx cancer SCC/Lymph node</td>
</tr>
<tr>
<td>8</td>
<td>55/F</td>
<td>CAM</td>
<td>8M</td>
<td>Weakness</td>
<td>-</td>
<td>1,331</td>
<td>Biceps/4–3</td>
<td>PSL</td>
<td>Uterine corpus cancer Adeno/Lymph node Prostate cancer Adeno/Bone, lung</td>
</tr>
<tr>
<td>9</td>
<td>72/M</td>
<td>CAM</td>
<td>4M</td>
<td>Rash</td>
<td>+</td>
<td>700</td>
<td>Biceps/4</td>
<td>PSL</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>57/F</td>
<td>Control</td>
<td>--</td>
<td>--</td>
<td>-</td>
<td>126</td>
<td>Biceps/5–4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>70/F</td>
<td>Control</td>
<td>--</td>
<td>--</td>
<td>-</td>
<td>84</td>
<td>Quadriceps/4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>17/M</td>
<td>Control</td>
<td>--</td>
<td>--</td>
<td>-</td>
<td>103</td>
<td>Quadriceps/5–4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>21/M</td>
<td>Control</td>
<td>--</td>
<td>--</td>
<td>-</td>
<td>57</td>
<td>Biceps/4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>36/M</td>
<td>Control</td>
<td>--</td>
<td>--</td>
<td>-</td>
<td>51</td>
<td>Biceps/5</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>


Subsequently, 0.2 mL of chloroform was added to each sample, followed by vigorous shaking for 15 s and centrifugation for 15 minutes at 4°C. Next, 0.5 mL isopropanol was added to each sample, followed by another centrifugation step for 15 minutes at 4°C. Isopropanol and 70% ethanol were used for RNA precipitation. We measured the RNA concentrations using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, USA) and evaluated the quality of total RNA using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA).

Microarray analysis
We used a SurePrint G3 Human GE 8×60k Microarray (Agilent Technologies) to evaluate the gene expression profiles. The SurePrint G3 microarray, which contains 27,958 Entrez gene targets and 7,419 long intergenic non-coding RNAs, was used according to the following protocol.

First, we synthesized Cy3-labeled complementary DNA (cDNA) from 100 ng of total RNA using a one-color Low Input Quick Amp Labeling kit (Agilent Technologies), according to the manufacturer’s instructions, followed by purification on an RNeasy column (Qiagen, Valencia, USA). Dye incorporation and complementary RNA (cRNA) yields were evaluated on a NanoDrop ND-1000 spectrophotometer. Subsequently, 0.6 μg of Cy3-labeled cRNA was fragmented at 60°C for 30 minutes in a reaction volume of 25 μL that contained 1x Agilent fragmentation buffer and 2x Agilent blocking agent, according to the manufacturer’s instructions. On completion of the fragmentation reaction, 25 μL of 2x Agilent hybridization buffer was added to the fragmentation mixture, which was subsequently hybridized to the Agilent SurePrint G3 Human GE 8×60k Microarrays for 17 h at 65°C in a rotating Agilent hybridization oven. After hybridization, the microarrays were washed for 1 minute at room temperature with GE Wash Buffer 1 (Agilent) and for 1 minute at 37°C with GE Wash buffer 2 (Agilent) and dried by brief centrifugation. The slides were immediately scanned after washing on an Agilent DNA Microarray Scanner (G2565CA) using the one-color scan setting for 8×60k array slides (scan area: 61 mm ×21.6 mm, scan resolution: 3 μm, dye channel: 100% Green PMT). The scanned images were analyzed using the Feature Extraction Software 10.10 program (Agilent) with the default parameters to obtain the background-subtracted and spatially detrended processed signal intensities.

Microarray data processing and analysis
The array data comprised 42,405 entities. The detection algorithm assigned a present, marginal, or absent call to each entity according to the feature extraction. We used the Gene Spring GX software program (Version 12.6; Agilent) to analyze the raw microarray data. The data were initially selected using the present and marginal detection values, designated as entities wherein at least 100.0% of samples in any one of the four groups (PM, DM, CAM, and Control) had flags in the present or marginal categories. We then performed a clustering analysis of all cases. The data were further filtered to determine fold changes in the expression relative to the Control group, and a GO analysis was performed to filter the entities.
Real-time qRT-PCR

Real-time qRT-PCR was used to synthesize cDNA from 1 μg of total RNA using an Oligo-dT primer (Promega, Madison, USA). The primers for each candidate exon were designed using the Primer3 software program (http://frodo.wi.mit.edu/primer3/input.htm). Real-time qRT-PCR was performed on an iCycler system (Bio-Rad Laboratories, Hercules, USA) with iQ CYBR Green Supermix (Bio-Rad). The reaction conditions were 95°C for 3 minutes and 40 cycles of 10 s at 95°C (denaturation), followed by 10 s at 55°C (annealing) and 30 s at 72°C (extension).

All experiments were performed in triplicate. The fluorescence emission spectra were continuously monitored and analyzed with a sequence detection software program. The expression of glyceraldehyde-3-phosphate dehydrogenase was simultaneously quantified as an internal standard control.

We identified eight genes related to the pathological condition from the GO results: Human Leucocyte Antigen (HLA)-ABC, HLA-DR, HLA-DQ, CD8, Intercellular Adhesion Molecule (ICAM)-1, Vascular Cell Adhesion Molecule (VCAM)-1, Myxovirus resistance protein (MX)-1, and Aquaporin (AQP) 4. These genes are all related to inflammatory pathology and muscle fiber damage and were used for the real-time qRT-PCR evaluation of the 14 initial cases. The expression ratio in each IM group was calculated relative to the Control group.

The following primers were used:

**HLA-ABC**
5’-GGCTCTGATGTCCTCCAAC-3’ (forward)
5’-CAAGTCAAAAGGGAAGGC-3’ (reverse)

**HLA-DR**
5’-CATGCAAAAGGCTCCAACC-3’ (forward)
5’-GTTGGGCTCTCAGTTCCA-3’ (reverse)

**HLA-DQ1**
5’-CCCTACCTTCCTCCCTCGT-3’ (forward)
5’-GAACTTCAGGCTCCAGTGT-3’ (reverse)

**CD8**
5’-GACGTGTTTGGCATAAGTC-3’ (forward)
5’-TTTGCAGCGGTAGACGT-3’ (reverse)

**ICAM-1**
5’-GTGACCGTAAATGCTCTTC-3’ (forward)
5’-CCTGAGTGCCCATTAGAC-3’ (reverse)

**VCAM-1**
5’-GGAAAGATGGCTGATCCT-3’ (forward)
5’-GATTCTGGGTGCTCCGAT-3’ (reverse)

**MX-1**
5’-TCGGCAACAGACTCTTCCAF-3’ (forward),
5’-AAAGGGATGTTGGCTTAGAT-3’ (reverse)

**AQP4**
5’-CGTGATCATGGCTCCTGGT-3’ (forward)
5’-TCAGTCGGTTGAACTACA-3’ (reverse).

**Immunohistochemistry**

**IHC procedures**
For 62 cases, an IHC analysis was performed using the standard avidin biotin-peroxidase complex method. The following primary antibodies were used: HLA-ABC (1:6,000, mouse IgG1; Becton-Dickinson, San Jose, USA), HLA-DR (1:300, mouse IgG1; Becton-Dickinson), HLA-DQ (1:300, mouse IgG1; Becton-Dickinson), CD8 (1:150, mouse IgG1; Dako, Glostrup, Denmark), ICAM1 (1:50, mouse IgG1; AbD Serotec, Raleigh, USA), VCAM1 (1:50, mouse IgG1; Becton-Dickinson), MX-1 (1:1,000, rabbit polyclonal; GeneTex, Irvine, USA), and AQP4 (1:200, rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, USA).

Serial 6 μm thick sections were air dried and fixed in ice-cold acetone for 3 minutes. The samples were then incubated with normal horse or goat sera (Vector Laboratories, Burlingame, USA) for 60 minutes, followed by incubation with the primary antibodies. After washing with phosphate-buffered saline, the sections were incubated with biotinylated horse anti-mouse IgG or biotinylated goat anti-rabbit IgG (Vector Laboratories) for 60 minutes. Next, all sections were washed extensively, exposed to an avidin-biotin complex (Vector Laboratories) for 60 minutes, and stained with diaminobenzidine for 10 minutes.

**IHC analysis**
The expression of the protein product of each gene was analyzed semiquantitatively. Overexpressed genes were evaluated on a scale of 0-4: 0 = no expression, 1 = expression in 1-25% of analyzed structures, 2 = expression in 26-50%, 3 = expression in 51-74%, and 4 = expression in 75-100%. Because the AQP4 expression was decreased in IM samples, the expression levels were analyzed as follows: 0 = normal expression, -1 = expression in 75-99% of analyzed structures, -2 = expression in 51-74%, -3 = expression in 26-50%, and -4 = expression in 0-25%.

**Statistical analysis**
The frequencies of each special protein product overexpressed or underexpressed in the fibers were expressed as the means ± one standard error. The three IM groups were compared using Scheffe’s test. The real-time qRT-PCR results between pairs of groups were compared using the t-test.

**Results**

In the clustering analysis of all 14 patients in a hierarchical dendrogram, the patients were divided into three groups: Control, PM, and DM + CAM. The PM group was distinct from patients in the original DM and CAM groups, whereas patients in the latter groups overlapped in the dendrogram (Fig. 1).

Using a cut-off value of a twofold difference from the Control expression level, we created Venn diagrams of the genes that met this criterion (Fig. 2). A total of 1,609 genes were upregulated in all three groups. A GO analysis detected 385 ontologies (p<0.01) reflective of a broad spectrum of inflammation, including immune system processes, humoral response, innate immune response, leukocyte activation, and cytokine-mediated signaling pathways (Table 2).
The 392 downregulated genes in all three groups reflected muscle fiber damage and homeostatic disorders.

Next, we performed a GO analysis to evaluate the specific gene expression profile of each group. In the PM group, 1,929 genes were specifically upregulated, corresponding to 45 ontologies (p<0.01) reflective of cell-mediated immune responses such as leukocyte activation, cell adhesion, and regulation of T-cell activation. In the DM group, 205 genes were specifically upregulated and 365 were downregulated. However, no ontologies were detected by the GO analysis (p>0.05). In the CAM group, 288 genes were specifically upregulated and 259 were downregulated; however, no ontologies were detected (Table 2).

We identified eight genes (HLA-ABC, HLA-DR, HLA-DQ, CD8, ICAM-1, VCAM-1, MX-1, and AQP4) from the GO analysis and evaluated their expression levels via real-time qRT-PCR in the original 14 patient specimens. In the microarray analysis, HLA-ABC was equally expressed in all groups. HLA-DR, HLA-DQ, CD8, ICAM-1, and VCAM-1 were overexpressed most strongly in the PM group, whereas MX-1 was expressed more strongly in the DM group. AQP4, which reflects muscle fiber damage, was strongly downregulated in the PM group (Table 3).

In the real-time qRT-PCR study, HLA-DR, HLA-DQ, CD8, ICAM-1, and VCAM-1 were overexpressed most strongly in the PM group. MX-1 was overexpressed in the DM and CAM groups, but not in the PM group. The AQP4 expression was strongly downregulated in the PM group (Fig. 3A-H1).

The IHC analysis yielded similar results to those from the microarray and real-time qRT-PCR analyses (Fig. 3A-H2,
Table 2.
a) Fold changes (vs. Control) in the microarray analysis.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>PM</th>
<th>DM</th>
<th>CAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-A</td>
<td>6.08 ± 3.11</td>
<td>7.13 ± 0.94</td>
<td>7.07 ± 1.66</td>
</tr>
<tr>
<td>HLA-B</td>
<td>8.28 ± 4.34</td>
<td>7.97 ± 1.52</td>
<td>8.14 ± 1.38</td>
</tr>
<tr>
<td>HLA-C</td>
<td>9.43 ± 4.89</td>
<td>9.56 ± 2.14</td>
<td>9.35 ± 1.64</td>
</tr>
<tr>
<td>HLA-DR8</td>
<td>16.67 ± 10.80</td>
<td>2.68 ± 1.32</td>
<td>2.63 ± 0.82</td>
</tr>
<tr>
<td>HLA-DRB1</td>
<td>10.18 ± 6.30</td>
<td>2.68 ± 1.32</td>
<td>2.15 ± 0.47</td>
</tr>
<tr>
<td>HLA-DQA1</td>
<td>14.25 ± 7.06</td>
<td>1.41 ± 0.37</td>
<td>1.11 ± 0.47</td>
</tr>
<tr>
<td>HLA-DQB1</td>
<td>29.69 ± 25.70</td>
<td>5.63 ± 3.77</td>
<td>1.83 ± 1.09</td>
</tr>
<tr>
<td>CD56</td>
<td>17.70 ± 12.42</td>
<td>2.84 ± 1.23</td>
<td>1.87 ± 1.15</td>
</tr>
<tr>
<td>CD8b</td>
<td>16.28 ± 13.46</td>
<td>4.88 ± 2.22</td>
<td>3.06 ± 2.10</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>10.93 ± 3.97</td>
<td>4.98 ± 1.30</td>
<td>5.27 ± 1.86</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>19.32 ± 9.29</td>
<td>8.38 ± 1.68</td>
<td>9.12 ± 3.05</td>
</tr>
<tr>
<td>MX-1</td>
<td>8.75 ± 0.54</td>
<td>58.04 ± 14.12</td>
<td>48.19 ± 39.27</td>
</tr>
<tr>
<td>AQP4</td>
<td>0.09 ± 0.06</td>
<td>0.59 ± 0.19</td>
<td>0.53 ± 0.18</td>
</tr>
</tbody>
</table>

b) The expression of pathology-related genes is variable.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>PM</th>
<th>DM</th>
<th>CAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC Class I</td>
<td>HLA-ABC</td>
<td>PM = DM = CAM</td>
<td></td>
</tr>
<tr>
<td>MHC Class II</td>
<td>HLA-DR</td>
<td>PM &gt;&gt; DM = CAM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HLA-DQ</td>
<td>PM &gt;&gt; DM = CAM</td>
<td></td>
</tr>
<tr>
<td>Lymphocyte/monocyte markers</td>
<td>CD8</td>
<td>PM &gt;&gt; DM = CAM</td>
<td></td>
</tr>
<tr>
<td>Adhesion molecules</td>
<td>ICAM-1</td>
<td>PM &gt; DM = CAM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VCAM-1</td>
<td>PM &gt; DM = CAM</td>
<td></td>
</tr>
<tr>
<td>Interferon-induced protein</td>
<td>MX-1</td>
<td>PM &lt;&lt; DM = CAM</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>AQP4</td>
<td>PM &lt;&lt; DM ≤ CAM</td>
<td></td>
</tr>
</tbody>
</table>

PM: polymyositis, DM: dermatomyositis, CAM: cancer-associated myositis

4). Major histocompatibility complex (MHC) class 1 and class 2 have an antigen-presenting function in the immune response.

CD8 plays an important role in cell-mediated immunity. ICAM-1 and VCAM-1 are well-known cell adhesion molecules involved in cell-to-cell interactions. MX-1 is an interferon α/β-inducible protein. AQP4 is a membrane protein belonging to a family of water channel proteins that facilitate water movement across biological membranes.

The expression of MHC class 1 (HLA-ABC) was high in all three groups. Notably, the expression levels of MHC class 2 (HLA-DR, HLA-DQ), CD8, and VCAM-1 were significantly higher in the PM group than those in the DM and CAM groups, and the ICAM-1 expression was also higher in the former group. In contrast, the DM and CAM groups had significantly higher levels of MX-1 and AQP-4 expression (p<0.01) than the PM group. Thus, the protein expression levels were similar to those detected via the gene expression analyses. Notably, in the PM group, inflammation-related factors and cell adhesion molecules were much more strongly expressed than those in the DM and CAM groups, whereas there were many similar tendencies in the expression and distribution between the latter two groups, making a distinction between these groups difficult.

Table 3. Results of a Gene Ontology (GO) Analysis Based on the Venn Diagrams.

A. Genes with common variations among the three groups
   GO of 1,609 upregulated genes
   385 GO (p < 0.01)
   immune system processes, humoral response, innate immune response, leukocyte activation, cytokine mediated signaling pathway… etc.
   GO of 332 downregulated genes
   13 GO (p < 0.05)
   muscle system processes, actin-myosin filament sliding, sarcomere, actin cytoskeleton… etc.

B. Genes with variations specifically in PM specimens
   GO of 1,292 upregulated genes
   45 GO (p < 0.01)
   immune response, leukocyte activation, cell adhesion… etc.
   GO of 951 downregulated genes
   79 GO (p < 0.01)
   cellular respiration, mitochondrion, sarcomere… etc.

C. Genes with variations specifically in DM specimens
   GO of 205 upregulated genes
   0 GO (p < 0.01)
   GO of 356 downregulated genes
   0 GO (p < 0.01)

D. Genes with variations specifically in CAM specimens
   GO of 288 upregulated genes
   0 GO (p < 0.01)
   GO of 259 downregulated genes
   0 GO (p < 0.01)

PM: polymyositis, DM: dermatomyositis, CAM: cancer-associated myositis

Discussion

We obtained similar results through microarray, real-time qRT-PCR, and IHC analyses of specimens from patients with IMs. We obtained three findings from this study: 1) DM and CAM are similar, 2) PM is different from DM and CAM, and 3) the gene expressions of CAM are similar, regardless of the type of cancer.

Many reports published in the last decade have described the application of microarray techniques in IM research (24-27). Although some reports have analyzed PM and DM, few have investigated CAM. In these previous reports, microarray techniques were useful for the correct classification of muscle diseases such as IMs, Duchenne muscular dystrophy, and nemaline myopathy (27). The expression of genes related to cytokines, MHC class 1 and 2 molecules, granzymes, adhesion molecules, and the actin cytoskeleton was found to be increased in cases of IM. These genes are differentially expressed depending on condition. Genes encoding cytokines, MHC class 1 and 2, and adhesion molecules are more strongly expressed in patients with PM and inclusion body myositis (IBM) than in patients with DM. However, genes induced by interferon α/β, such as Interferon Stimulated Gene (ISG)-15 and MX-1, are more strongly expressed in patients with DM than in patients with PM and IBM (28).

In the present study, we expanded the target of the mi-
Correlation of the microarray data from PM and DM to CAM. We newly found that DM and CAM belong to the same group in a hierarchical dendrogram and have similar tendencies in the expression of pathology-related genes according to the GO analysis. On the other hand, PM was separated from other DM, CAM, and Control in the hierarchical dendrogram. According to the results of the GO analysis, DM and CAM had no significant ontologies, whereas PM was associated with strong inflammation.

The MHC class I expression on the sarcolemma, which is absent in normal muscle fibers, is upregulated in patients with IMs (29-31). Additionally, MHC class 2 is not expressed on normal myofibers, and some investigators have detected this antigen in specimens from patients with IMs (32-35). HLA-DR was overexpressed on the sarcolemma in the PM group, while weakly expressed in the DM and CAM groups in the present study. IHC staining with an HLA-DQ antibody revealed a similar pattern that confirmed the microarray data.

CD8 is overexpressed on inflammatory cells in patients with PM. In the present study, CD8 was significantly overexpressed on inflammatory cells in the PM group compared with the DM and CAM groups.

VCAM-1 and ICAM-1 are associated with IMs, and a previous report described significantly upregulated ICAM and VCAM expression levels on blood vessels and muscle fibers in patients with IMs relative to controls, which exhibited a weak or absent expression (36-38). The ICAM-1 and VCAM-1 expression was significantly stronger on the sarcolemma and blood vessels in the PM group than that in the DM and CAM groups in the present study.

Greenberg et al. observed MX-1 via dense perifascicular staining and occasional staining of all myofibers and capillaries in patients with DM (28). In our study, MX-1 was overexpressed on some myofibers and capillaries, mainly perifascicular, in the DM group, whereas in was only over-

Figure 3. Real-time qRT-PCR and immunohistochemistry (IHC) results. A-H-1: Real-time qRT-PCR analysis results. Relative Expression indicates the gene expression in inflammatory myopathies (IMs) relative to the Control. *p<0.05 vs. Control A-H-2: IHC staining grades. The following staining scale was used. Overexpressed genes: 0=no expression, 1=expression in 1%-25% of the analyzed structures, 2=expression in 26%-50%, 3=expression in 51%-74%, and 4=expression in 75%-100% AQP4 expression was decreased in IMs and was graded according to the following scale: 0 normal expression, 1=expression in 75%-99%, 2=expression in 51%-74%, 3=expression in 26%-50%, and 4=expression in 0%-25%, *p<0.05; **p<0.01.
Figure 4. Photomicrographs of polymyositis (PM), dermatomyositis (DM), cancer-associated myositis (CAM), and Control specimens. Immunohistochemical reactions specific for HLA-ABC (A), HLA-DR (B), HLA-DQ (C), CD8 (D), ICAM-1 (E), VCAM-1 (F), MX-1 (G), and AQP4 (H) in PM, DM, CAM, and Control. Magnification, 200×.

expressed in some CAM group cases. Therefore, these results could suggest similarities between the DM and CAM groups.

AQP4 is expressed in healthy brain and muscle tissues (39) and well known to associate with neuromyelitis optica. The AQP4 expression is also reduced in the skeletal muscle in patients with Duchenne muscular dystrophy, Fukuyama-type congenital muscular dystrophy, and sarcoglycanopathy (39-41). In the present study, the IHC analysis revealed a decrease in the AQP4 expression in both regenerating fibers and many non-necrotic fibers in patients with IMs, particularly PM. This finding indicates that in IMs, a
decrease in the AQP4 expression in the skeletal muscle causes a functional defect in skeletal muscle fibers that reflects severe inflammation of the skeletal muscle.

Another previous report reviewed the muscle biopsy findings in patients with PM and DM with or without malignancy (42). The author concluded that the incidence of rare-infiltrative type muscle pathology may be a predictive marker of DM with malignancy. In our IHC analysis of many cases, CAM was closely similar to DM, regardless of cancer variation.

In addition, we found that MX-1 was strongly expressed in not only DM, but also CAM, as discussed above. In our study, the most common site-specific malignancy was lung cancer, and the most common pathological type was adenocarcinoma. Although various types of malignancies have been reported to be associated with both DM and PM, the major types of malignancy vary depending on the study population and region. In a previous study conducted by Hill et al. in Sweden, Denmark, and Finland, the most common malignancy was International Classification of Disease (ICD)-7 code 162 or lung, trachea, and bronchus cancers (12). Other studies conducted in Western countries have reported adenocarcinoma of the ovary, lung, or gastrointestinal tract (11, 16, 43), whereas studies in Southeast Asia, Southern China, and Northern Africa reported high rates of nasopharyngeal carcinoma (16, 44-46). Regarding the pathological type of malignancy, another study concluded that adenocarcinomas, including lung, ovarian, cervical, gastric, and pancreatic cancers, were most commonly associated with DM (47). Our present data confirmed the association of this classification using gene expression profiling.

Considering the results of the present study, inflammation of PM was mainly mediated by the cellular immune system, and we speculate that the muscle fiber is the main inflammatory site in this condition. In contrast, DM and CAM did not show strong inflammation on muscle fibers and may thus exhibit similar humoral immune system-related pathologies. Additionally, these findings may indicate that a malignancy can remotely induce muscle inflammation and that the etiologies of CAM and DM share similar features. Our study may therefore provide a key to understanding the etiology of CAM and the clinical importance of cancer screening in patients with IMs, particularly DM. Further studies are needed to confirm these findings.

The authors state that they have no Conflict of Interest (COI).

References


The Internal Medicine is an Open Access article distributed under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License. To view the details of this license, please visit (https://creativecommons.org/licenses/by-nc-nd/4.0/).