Original Paper

Deep RNA sequencing of muscle tissue reveals absence of viral signatures in dermatomyositis

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Additional resources and electronic supplementary material: supplementary material

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Abstract

Objective: To explore a possible connection between active viral infections and manifestation of dermatomyositis (DM).
Methods: Skeletal muscle biopsies were analyzed from patients diagnosed with juvenile (n=10) and adult (n=12) DM. Adult DM patients harbored autoantibodies against either TIF-1γ (n=7) or MDA5 (n=5). Additionally, we investigated skeletal muscle biopsies from non-diseased controls (NDC, n=5). We used an unbiased high-throughput RNA sequencing (HTS) approach to detect viral sequences. To further increase sequencing depth, a host depletion approach was applied.
Results: In this observational study, no relevant viral sequences were detected either by native sequencing or after host depletion. The absence of detectable viral sequences makes an active viral infection of the muscle tissue unlikely to be the cause of DM in our cohorts.
Discussion: Type I interferons (IFN) play a major role in the pathogenesis of both juvenile and adult DM. The IFN response is remarkably conserved between DM subtypes classified by specific autoantibodies. Certain acute viral infections are accompanied by a prominent type I IFN response involving similar downstream mechanisms as in DM. Aiming to elucidate the pathogenesis of DM in skeletal muscle tissue, we used deep RNA sequencing and a host depletion approach to detect possible causative viruses.

Keywords: Dermatomyositis (DM), Interferon (IFN), Viral signature, Next generation sequencing

Introduction

Although the pathogenesis of DM is not completely understood, it is well established that type I IFN plays a key role in both juvenile and adult DM. The IFN response can be defined by a specific up-regulation of IFN-stimulated molecules such as ISG15 or MxA and is comparable to that found in lupus erythematosus (SLE) and certain inherited interferonopathies, but also in viral infections [10]. In fact, parvovirus B19, coxsackie virus, polyomavirus, Epstein-Barr virus (EBV), influenza virus, human immunodeficiency virus (HIV), and SARS-CoV-2 have been associated with DM onset [3]. Since the detection of specific autoantibodies, such as anti-Mi-2, anti-TIF-1γ, anti-NXP2, anti-SAE, and anti-MDA5, the spectrum of DM can be defined more precisely regarding prognosis and clinical course such as risk of cancer development [27]. DM subtype-specific investigation of type I IFN-regulated transcripts identified a set of significantly dysregulated genes in muscle biopsies derived from anti-TIF-1γ+ patients [4]. Moreover, MDA5, RIG1, and TRIM33 (TIF1γ) are specifically involved in downstream signaling of viral infections [13]. MDA5 is a key protein sensor for viral double-stranded RNA (dsRNA) motifs to induce expression of IFN1 in certain viral diseases [6], and TRIM33 inhibits endogenous retrovirus (ERV) gene transcription [21]. Recent studies have demonstrated that myositis autoantibodies, most commonly anti-TIF-1γ, anti-NXP2, and anti-MDA5, are also found in more than 50% of juvenile myositis patients, with different frequencies in different populations, but again associated with specific clinical manifestations and prognosis [12, 15, 22, 29].

It is well described, especially in children, that DM can occur with an acute onset of general viral infection-like symptoms such as fever, fatigue, and apathy [20].

Hence, the aim of our project was the identification of any viral signatures (in terms of an active virus replication) in skeletal muscle samples using an unbiased high-throughput sequencing approach. In contrast to other targeted approaches such as virus-specific PCRs, we aimed at viral genome detection without prior restrictions to probable pathogens to investigate any - including previously unknown - possible viral species.

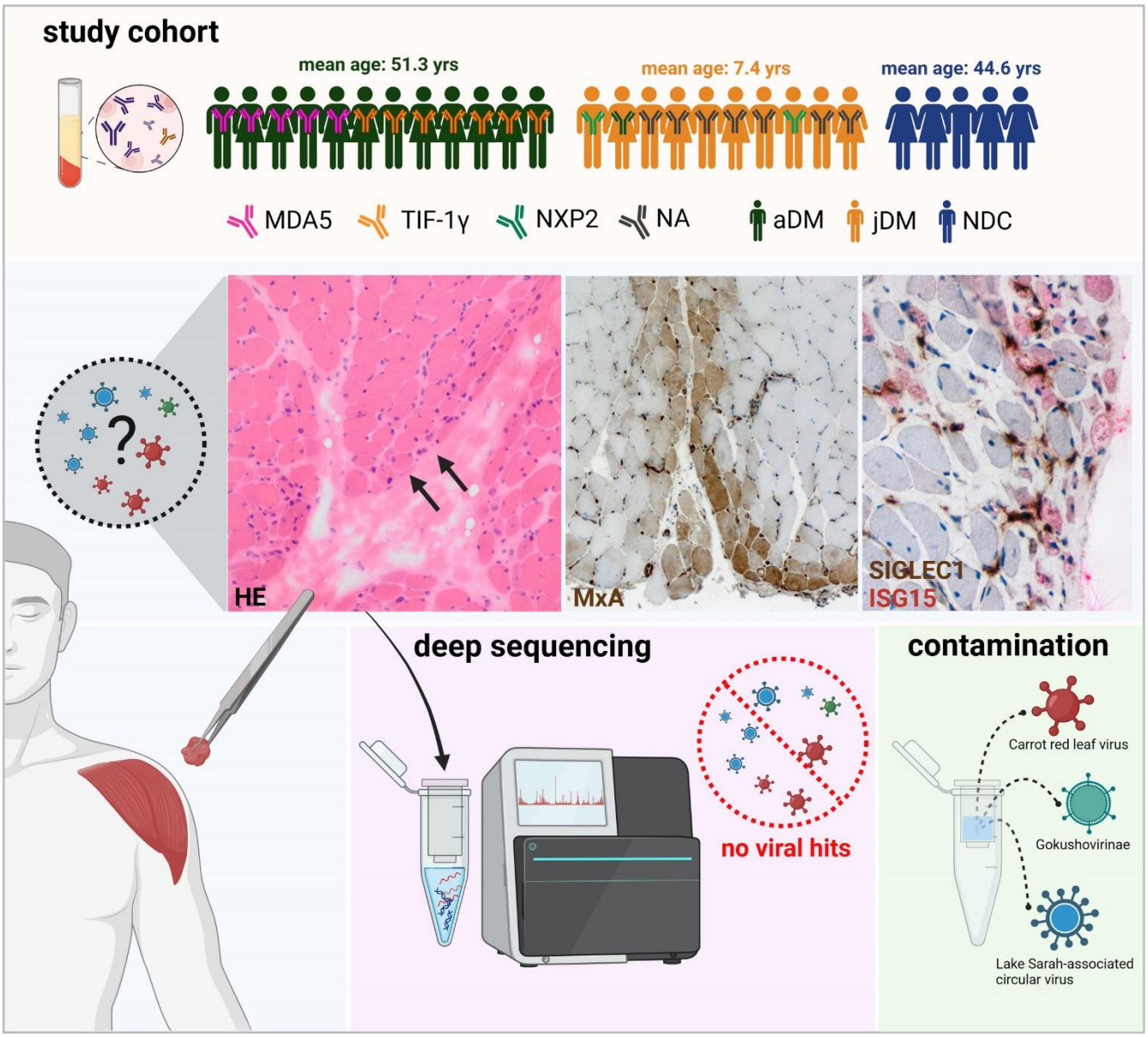

Figure 1: Experimental study design. The study cohort, including subgroups and information on autoantibodies, is given in the upper panel. Histological work-up of DM skeletal muscle samples shows perifascicular atrophy (HE, arrows). Immunohistochemical staining of type I IFN-inducible proteins ISG15 and MxA demonstrates a perifascicular staining pattern with SIGLEC1-positive macrophages (brown) in close proximity to ISG15 positive muscle fibers (red). Deep sequencing of DM patients' skeletal muscle biopsies only revealed viral reads derived from column-based extraction and library preparation kit contaminations. (aDM: adult dermatomyositis, jDM: juvenile dermatomyositis, NDC: non-diseased controls, NA: No autoantibody testing, yrs: years).

Material and Methods

Patient cohort

Skeletal muscle biopsies were analyzed from treatment-naive patients diagnosed directly after symptom onset with juvenile or adult DM according to ENMC diagnostic criteria [14]. We included n=22 DM cases from three institutions between 2008 and 2018, in which the serum of adult patients was either positive for autoantibodies against TIF-1γ (n=7) or MDA5 (n=5). The morphological muscle involvement was related to the underlying autoantibody with perifascicular atrophy and inflammation in TIF-1γ+ patients and less severe muscle damage in MDA5+ patients [1]. The serum of n=2 juvenile patients was positive for autoantibodies against NPX2. The other juvenile patients were diagnosed before autoantibody testing was widely available

and retroactive information on autoantibodies was not available. Additionally, we investigated skeletal muscle biopsies (n=5) from non-diseased controls (NDC) with nonspecific complaints, without overt muscle weakness, absence of any morphologic abnormalities in the skeletal muscle biopsies, elevated creatine kinase (CK) levels, or laboratory evidence of any systemic inflammation including negativity of autoantibody testing. Informed consent was obtained from all patients at each institution involved. Procedures were approved by the official ethical standards committee (EA2/163/17) at the Charité – Universitätsmedizin Berlin. The experimental setup is shown in **Figure 1**.

High throughput-sequencing of RNA

Total RNA was extracted from fresh-frozen, cryopreserved (at -80°C) skeletal muscle specimens using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. RNA extraction was checked by testing for Beta-2 microglobulin (B2M) mRNA using a RT-qPCR as previously described [23]. For the detection of viral sequences two strategies were applied: (i) unbiased native sequencing of the nucleic acids and (ii) a more sensitive sequencing approach after the specific removal of ribosomal RNA (rRNA). The detailed steps are given in **Supplementary material and methods**.

Statistics and HTS data analyses

For virus detection, we applied two approaches for viral read identification. First, a computational pipeline that consists of a collection of bash shell (C. Ramey, GNU bash, available at https://www.gnu.org/software/bash/) scripts that invoke third-party and custom in-house programs. Overall execution is coordinated by a Slurm Pipeline Python package (T. C. Jones, Slurm Pipeline, available at https://github.com/acorg/slurm-pipeline). Second, we classified sequences with Kraken2 (version 2.1.3) and the Kraken2 [28] 'standard' database (available at https://benlangmead.github.io/aws-indexes/k2, revision date 10/9/23). The results were visualized using KRONA [19] and Pavian [5] and independently analyzed by two virologists. No special cut-off value was applied, but rather each individual virus hit was examined and evaluated individually. The detailed steps for both analyses are given in **Supplementary material and methods**.

Data availability

The sequencing data (non-human reads) of the samples generated in this study have been deposited in the Sequence Read Archive (SRA) under the accession numbers SAMN39051134 - SAMN39051187.

Results

Sequencing results before and after rRNA removal

Successful RNA extraction was confirmed by the detection of beta-2-microglobulin mRNA in all samples (**Supplementary table 1**). All samples were processed in the two sequencing approaches: Using native sequencing, a total of 661.6 Mio reads (Range: 7.1-32.8 Mio per sample, mean: 24.5 Mio, median: 25.5 Mio) were generated for all patients analyzed. Roughly, 90% of the resulting reads could be mapped to rRNA references (NR_003287.1, NR_145820.4) and less than 1% of the reads mapped against a globin reference sequence (HBA1: NM_000558.5, HBA2: NM_000517.6, HBB: NM_000518.5, HBD: NM_000519.4) (**Table 1**).

For removal of rRNA and globin sequences, we initially applied the QIAseq FastSelect -rRNA and Globin HMR Kit for four samples. This resulted in a nearly complete removal of rRNA (<1% remaining) and a similar reduction of globin sequences (data not shown). As the initial number of globin mRNA reads was already low, we proceeded with the removal of only rRNA for all samples.

After rRNA depletion, a total of 558.3 Mio reads (Range: 5.3-38.6 Mio per sample, mean: 20.7 Mio, median: 20.5 Mio) were generated. A maximum of 5.69% rRNA reads and well below 1% globin reads remained.

As expected, Kraken2 classification of reads from both approaches resulted in many reads classified as being of human, bacterial, or archaea origin (**Supplementary table 2**). Although not within the scope of the present study, which focuses on viruses, the detection of these reads can be used to ensure consistent quality and comparability of the sequencing data for all samples. The matched bacterial reads mainly correspond to environmental bacteria and are likely explained by contamination from the environment or the skin microbiome (e.g. Cutibacterium acnes).

Table 1. Overview of samples, PCR, and sequencing results. The table includes number of generated reads and proportion of rRNA and globin reads for native sequencing and sequencing after rRNA depletion.

				Native sequencing					rRNA removal				
Patient ID	**Cohort**	⚥	**age**	**Reads [R1+R2]**	**reads against rRNA***	**% rRNA**	**reads against Globin****	**% globin**	**Reads [R1+R2]**	**reads against rRNA***	**% rRNA**	**reads against Globin****	**% globin**
1	MDA 5	m	34	25,451,212	23,454,672	92.2	1	0.000004	23,752,226	700,270	2.95	2,965	0.012483
2	MDA 5	f	32	16,964,910	15,449,997	91.1	100	0.000589	11,763,512	115,577	0.98	116	0.000986
3	MDA 5	f	39	25,805,868	24,592,381	95.3	192	0.000744	23,635,252	349,242	1.48	54,525	0.230694
4	MDA 5	m	31	27,640,030	26,338,410	95.3	97	0.000351	23,982,524	530,301	2.21	1,507	0.006284
5	MDA 5	f	38	26,560,152	24,391,028	91.8	6.780	0.025527	12,321,160	142,752	1.16	269	0.002183
6	TIF-1γ	f	72	24,181,444	22,604,310	93.5	101	0.000418	32,040,902	1,823,768	5.69	388	0.001211
7	TIF-1γ	m	69	25,030,838	22,695,134	90.7	102	0.000407	32,634,054	839,161	2.57	1,214	0.003720
8	TIF-1γ	f	47	27,551,144	25,511,050	92.6	11	0.000040	37,285,322	877,105	2.35	225	0.000603
9	TIF-1γ	f	64	26,854,504	22,726,155	84.6	53	0.000197	38,565,498	969,885	2.51	3,156	0.008183
10	TIF-1γ	f	38	27,316,894	24,417,085	89.4	6	0.000022	19,416,276	233,351	1.20	293	0.001509
11	TIF-1γ	f	83	27,684,124	24,577,672	88.8	122	0.000441	38,328,838	681,366	1.78	548	0.001430
12	TIF-1γ	m	69	29,464,600	27,636,300	93.8	83	0.000282	21,102,676	388,385	1.84	22	0.000104
13	NDC	f	70	30,284,074	26,373,635	87.1	55	0.000182	28,112,926	388,141	1.38	9,949	0.035389
14	NDC	f	51	25,886,910	25,146,497	97.1	0	0.000000	25,898,230	711,277	2.75	332	0.001282
15	NDC	m	53	22,971,962	20,643,167	89.9	590	0.002568	6,997,044	33,460	0.48	233	0.003330
16	NDC	f	17	32,842,356	28,922,129	88.1	34	0.000104	20,534,850	363,574	1.77	107	0.000521
17	NDC	f	32	19,838,176	18,580,234	93.7	532	0.002682	14,686,946	338,501	2.30	1,310	0.008919
18	jDM	m	20	26,934,130	25,226,685	93.7	10	0.000037	10,881,868	421,412	3.87	257	0.002362
19	jDM	f	15	24,941,254	22,494,587	90.2	98	0.000393	28,547,838	314,633	1.10	3,078	0.010782
20	jDM	m	2	21,805,986	19,930,688	91.4	22	0.000101	5,877,104	17,646	0.30	13	0.000221
21	jDM	f	2	22,330,238	20,058,021	89.8	284	0.001272	6,781,752	117,288	1.73	99	0.001460
22	jDM	m	6	7,149,280	4,980,683	69.7	26	0.000364	5,332,966	236,384	4.43	119	0.002231
23	jDM	f	5	23,297,262	21,965,327	94.3	62	0.000266	13,150,292	128,447	0.98	101	0.000768
24	jDM	m	7	23,303,882	21,816,633	93.6	59	0.000253	11,615,248	31,920	0.27	111	0.000956
25	jDM	m	10	20,889,528	19,197,167	91.9	13	0.000062	12,207,356	468,646	3.84	68	0.000557
26	jDM	m	2	19,364,716	16,680,717	86.1	141	0.000728	32,487,164	53,126	0.16	2,411	0.007421
27	jDM	f	5	29,292,446	27,326,249	93.3	816	0.002786	20,397,658	386,630	1.90	6,248	0.030631

* NR_003287.1, NR_145820.4
** HBA1: NM_000558.5, HBA2: NM_000517.6, HBB: NM_000518.5, HBD: NM_000519.4

Matches against virus sequences

For both sequencing approaches and both sequence classification approaches, no relevant human virus findings were detected. Both classification approaches resulted in overall similar results (**Supplementary figure 1**). A common finding were retroviral reads (Murine leukemia virus) which were previously described as common contamination from library preparation kit reagents [8]. Remarkably, after rRNA removal, a higher number of previously described contaminations from extraction columns and library preparation kit reagents were found that were not detected in the native sequencing approach. These mainly included plant- and algae-infecting viruses such as Carrot red leaf virus, Lake Sarah-associated circular virus, and Gokushovirinae. In one sample, viral reads against Kadipiro virus were identified, which was also previously shown to be a potential kit contamination [11, 18]. Additionally, we detected Circovirus-like viral reads that were previously associated with RNeasy MinElute columns used for RNA extraction [2]. In all depleted samples, including the control subjects, we found reads mapping against two short (<150 bp) genome regions of AAV-5, which we therefore did not count as a virus finding. We were not able to identify the origin of these two short sequences, but an origin in the used kits or reagents are also likely. Although only RNA library preparation was performed, it was also possible to detect DNA viruses, e.g., via transcripts or replication intermediates as shown by the detection of the DNA virus Gokushovirinae, Cycloviruses, and Chrysochromulina ericina virus.

Discussion

The aim of the project was to explore a putative viral pathogenesis of DM using an unbiased methodology. This was based on obvious evidence concerning molecular links between relevance and presence of IFN-related gene expression, and known autoantibody functions such as MDA5/RIG1 and TRIM33, as well as epitope homology of TRIM proteins with specific viral species including poxviruses [16].

Here, we detected viral sequences, especially after rRNA removal, in most of the samples. Nevertheless, the few viral reads concerning Murine leukemia virus, Carrot red leaf virus, Lake Sarah-associated circular virus, Gokushovirinae, Parvovirus NIH-CQV, and Kadipiro virus are very likely derived from column-based extraction and library preparation kit contamination [2] instead of being the cause of direct viral muscle infections, which have been reported for influenza viruses, enteroviruses, HIV, and hepatitis viruses [7, 17, 25] among others.

The increase in such matches in the rRNA-depleted samples indicates the higher sensitivity of this approach and that the sequencing depth was sufficient to detect potential causative virus sequences including RNA transcripts from active replicating DNA viruses, had they been present in the muscle samples. Therefore, it can be assumed that no viral pathogens were present at the times and sites of sample collection.

However, the absence of evidence of active viral infection in the muscle tissues at the time of sampling does not preclude previous viral infections.

Autoimmunity and the detection and accumulation of antibodies are well established to be at the root of the pathogenesis of DM [9]. Viral infections and virus exposure in turn contribute to accumulation of virus-specific antibodies and generation of autoantibodies, which may lead to autoimmunity via several pathways including molecular mimicry, epitope spreading, and bystander activation even after complete viral clearance [9, 24]. Multiple other factors such as genetic predisposition, host immune response, and viral strain may also play an important role in disease occurrence, progression, and prognosis. As antibodies recognizing viruses demonstrate a high abundance in DM patients, further studies using next generation sequencing techniques will be needed to investigate the viral exposure patterns in DM patients at different time points during the course of the disease [16, 26]. This may also help to detect new, previously unknown DM-specific autoantibodies to further prognostically stratify DM patients.

Author contributions

Werner Stenzel, Corinna Preusse, Victor Corman, and Josefine Radke designed the study concept, performed data analysis, and wrote the manuscript. Terry C. Jones and Julia Melchert performed data analysis and revised the manuscript. Randi Koll performed data analysis. Olivier Benveniste, Ulrike Schara-Schmidt, and Sarah Leonard-Louis provided muscle biopsy samples and revised the manuscript. Christian Drosten and Hans-Hilmar Goebel revised the manuscript and contributed to data analysis.

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Conflicts of interest

The authors declare no conflict of interest.

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