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ORIGINAL RESEARCH

The expression of gene *ANKRD1* correlates with hypoxia status in muscle biopsies of treatment-näive adult dermatomyositis

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OBJECTIVES. The ANKRD1 gene codes for the ankyrin repeat domain containing protein 1 and has an important role in myogenesis and possibly also in angiogenesis. Microvasculopathy is a cornerstone and an early pathological marker of change in dermatomyositis, leading to hypoxia and muscle perifascicular atrophy. These alterations could upregulate genes involved in myogenesis and angiogenesis such as ANKRD1. Therefore, we analyzed ANKRD1 expression in muscle biopsies of dermatomyositis and correlated with other hypoxia parameters and with histological changes.
METHODS. Total RNA was extracted from frozen muscle biopsies samples of 29 dermatomyositis patients. A control group consisted of 20 muscle biopsies from adult patients with non-inflammatory myopathy diseases. The gene coding for hypoxia-inducible factor 1, alpha subunit (HIF1A), was analyzed to estimate the degree of hypoxia. ANKRD1 and HIF1A transcript expression levels were determined by quantitative real time PCR.
RESULTS. Significantly higher ANKRD1 and HIF1A expression levels were observed in dermatomyositis relative to the control group ($P<0.001$, both genes). In addition, ANKRD1 and HIF1A were coexpressed ($r=0.703$, $P=0.001$) and their expression levels correlated positively to perifascicular atrophy ($r=0.420$, $P=0.023$ and $r=0.404$, $P=0.030$, respectively).
CONCLUSIONS. Our results demonstrate ANKRD1 overexpression in dermatomyositis correlated to HIF1A expression and perifascicular atrophy. ANKRD1 involvement in myogenesis and angiogenesis mechanisms indicates that further investigation is worthwhile.

KEYWORDS: Dermatomyositis; hypoxia; myogenesis; perifascicular atrophy; RNA expression.

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INTRODUCTION

The ankyrin repeat domain 1 (ANKRD1) is a transcription cofactor that translocates to the nucleus in response to mechanical stretch.[1] It is present not only in the nucleus but also in the I-band region of the sarcomere as a member of the titin-N2A mechanosensory unit[1] and interacts with myopalladin.[2] Moreover, the interaction of ANKRD1 and myopalladin is required for the maintenance of sarcomeric integrity.[2]

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ANKRD1 is present in fetal skeletal muscle fibers[3] and its expression decreases after birth, being scarcely expressed in the normal skeletal muscle of adults.[3-5] This expression profile in early development suggests potential functional roles of ANKRD1, as in cardiogenesis and myogenesis. The up-regulated production of ANKRD1 in adult muscles has been associated with pathological conditions such as denervated skeletal muscle,[4-6] myopathies,[5,7-9] muscle hypoxia;[10] it has also been associated to exercise.[11-15] ANKRD1 overexpression was also observed during experimental wound healing, with neovascularization and increased blood perfusion, also suggesting its role in angiogenesis.[16]

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Dermatomyositis (DM) is a systemic idiopathic inflammatory myopathy characterized by a subacute onset and proximal symmetric muscle weakness. The disease is also associated with cutaneous manifestations, including heliotrope and Gottron's papules.[17] The cornerstone of the DM pathogenesis involves vascular disturbances that lead to hypoxia, capillary necrosis and muscle perifascicular atrophy.[17,18]

Therefore, the hypoxic condition in muscle tissue leading to perifascicular atrophy found in DM may also upregulate ANKRD1 expression, which has been related to myogenesis and/or angiogenesis. To test this hypothesis, we analyzed *ANKRD1* expression in treatment-naïve skeletal muscle biopsies of adult DM and correlated this expression to skeletal muscle alterations due to hypoxia.

MATERIALS AND METHODS

Study design

The present retrospective study was performed at one center and included 29 drug therapy naïve DM patients and 20 adult controls (free of evidence of inflammatory myopathies), who were submitted to routine diagnostic skeletal muscle biopsies of the brachial biceps. The patients fulfilled at least four of the five Bohan and Peter criteria items,[19] and they were initially seen at the Myopathies Unit of our tertiary service. We excluded patients with (i) clinically amyopathic DM, (ii) myositis associated to overlap syndromes or to neoplasia, and (iii) necrotizing myopathies. The study was approved by our University Hospital Research Ethics Committee (case # 01445312.3.0000.0068).

Patient data

All the participants underwent a clinical evaluation and all available information was extensively reviewed. The following data were collected at the time of the muscle biopsy procedure: demographics (age, gender and ethnicity), clinical (disease duration) and laboratory data (serum level of creatine phosphokinase and aldolase). Serum level of creatine phosphokinase (normal range: 24 - 173 IU/L) and aldolase (normal range: 1.0 - 7.5 IU/L) levels were determined by the automated kinetic method. Limb muscle strength was graded according to the Medical Research Council Scale as **grade 0**: absence of muscle contraction; **grade I**: slight signs of contractility; grade II: movements of normal amplitude but not against gravity; **grade III**: normal range of motion against gravity; **grade IV**: full mobility against gravity and some degree of resistance; **grade V**: complete mobility against gravity and strong resistance.[20]

Total RNA extraction and cDNA Synthesis

Total RNA was extracted from muscle tissues using the RNeasy Fibrous Tissue Mini Kit (Qiagen Inc, Hilden, Germany). Evaluation of RNA quantification and purification was carried out by measuring absorbance and A260/A280 ratios, and ranges of 1.8 - 2.0 were considered satisfactory for purity standards. Denaturing agarose gel electrophoresis was used to assess the quality of the samples. Synthesis of cDNA was performed by reverse transcription from 1 μg total RNA, previously treated with 1 unit of DNase I (FPLC-pure, GE Healthcare, Piscataway, NJ), using random and oligo(dT) primers, RNase inhibitor and SuperScript III reverse transcriptase (Thermo Fisher Scientific, Carlsbad, CA), all according to the manufacturer's recommendations. The resulting cDNA was then treated with 1 unit of RNase H (GE Healthcare). For 200-500 ng of total RNA, the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was utilized. Ten μL of total RNA was reverse transcribed using MultiScribe™ Reverse Transcriptase according to the manufacturer's instructions. All cDNA samples were diluted with TE buffer, and stored at - 20 °C until later use.

Quantitative real time (qRT-PCR)

The expression levels of *ANKRD1* and *HIF1A* (hypoxia-inducible factors 1-alpha) were determined by qRT-PCR using the SYBR Green approach, in duplicate. Quantitative data were normalized relative to the geometric mean expression of three internal housekeeping control genes: hypoxanthine guanine phosphoribosyl transferase 1 (*HPRT*), ribosomal protein L41 (*RPL41*) and heterogeneous nuclear ribonucleoprotein K (*HNRPK*). Primer sequences were as follows (5´-3´): ANKRD1 F: GAGTGCGCGGAGCATCTTA, ANKRD1 R: GTCTCACCGCATCATGCAA, HIF1A F: CATCCAAGAAGCCCTAACGTGT, HIF1AR: CATTTTTCGCTTTCTCTGAGCAT, HPRT F: TGAGGATTTGGAAAGGGTGT, HPRT R: GAGCACACAGAGGGCTACAA, RPL41 F: GCGCCATGAGAGCCAAGT, RPL41 R: CTCCACGGTGCAACAAGCTA, HNRPK F:GAGCCCATCAGAATGGCAGAT, HNRPK R: AAGATCACCATATGAGCCACGA; these were synthesized by IDT (Integrated DNA Technologies, Coralville, IA). Sybr Green I amplification mixtures (12 μL) contained 3 μL of cDNA, 6 μL of 2x Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific), and forward and reverse primers. Reactions were run on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The cycle conditions comprised incubation at 50 °C for 2 min to activate UNG, initial denaturation at 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The

minimum concentration of primers was determined by the lowest threshold cycle (Ct) and maximum amplification efficiency while minimizing non-specific amplification (at final concentration of 200 nM for *ANKRD1*, *HIF1A*, *HPRT* and *HNRPK*, and 100 nM for *RPL41*). Analysis of DNA melting curves demonstrated a single peak for the whole set of primers. Standard curves were analyzed to check the efficiency of amplification of each gene. Additionally, the size of the amplified PCR products was checked by agarose gel electrophoresis. The equation $2^{-\Delta\Delta CT}$ was applied to calculate the relative expression of DM samples, where ΔCt = mean Ct *ANKRD1* - Ct geometric mean of housekeeping genes and ΔΔCt = ΔCt *ANKRD1* - mean ΔCt controls.[21]

Histological muscle biopsy analysis

Sequential frozen sections were first stained for hematoxylin-eosine (HE), and then by immunohistochemistry. Each muscle biopsy specimen was coded and analyzed separately. The presence of perifascicular atrophy was assessed semi-quantitatively. A visual analogue scale (VAS) was also included to score global degree of muscle inflammatory and abnormality from 0 (no inflammation or no abnormality) to 10 (most inflammation or most abnormal).

Immunofluorescence analysis

Immunofluorescence staining was performed to analyze *ANKRD1* expression in muscle biopsies. The mouse polyclonal antibody against human ANKRD1 (ab88661, Abcam, Cambridge, MA, 1:13) was used. Serial frozen sections of 5 µm - thickness were fixed for 10 min with methanol and acetone (1:1). Membranes were permeabilized with 0.1 % Triton X-100 and unspecific sites were blocked with 2 % bovine serum albumin (BSA). The primary antibody was incubated overnight at 4°C; the secondary antibody - goat anti-mouse IgG-Alexa Fluor (Thermo Fisher Scientific) was incubated for one hour. Nuclei were stained with DAPI (Thermo Fisher Scientific). Staining structures were scanned using a Zeiss LSM 510 Meta Confocal microscope (Carl Zeiss Inc, Thornwood, NY). Human cardiac muscle was used as a positive control for the reactions.

Statistical analysis

The Kolmogorov-Smirnov test was used to evaluate the distribution of each parameter. The demographic, clinical and laboratory features are expressed as mean and standard deviation (SD) for continuous variables or as frequencies and percentages for categorical variables. The median (25th - 75th percentile) was calculated for continuous variables not normally distributed. Comparisons between *ANKRD1* and *HIF1A* relative expression levels of patients and control groups, as well as DM relative expression levels and the patients laboratory and histological parameters were analyzed using Student's *t*-test or the Mann-Whitney test. Chi-squared or Fisher's exact test was used to evaluate the categorical variables. The correlations between the parameters were analysed by Spearman correlation. All of the analyses were performed using the SPSS 15.0 statistics software (Chicago, , IL,USA). Values of $P < 0.05$ were considered statistically significant.

RESULTS

The present study included 29 dermatomyositis patients (6 males and 23 females, mean age 51.2 ± 17.4 years) and 20 adult control individuals (5 males and 15 females, mean age 50.4 ± 15.5 years). The patients' demographic, clinical, laboratory and muscle biopsy features are presented in Table 1.

Higher *ANKRD1* and *HIF1A* expression levels were observed in samples of DM patients vs. controls ($P = 0.0002$ and $P < 0.0001$, respectively), as shown in Figure 1. *ANKRD1* relative expression correlated positively to *HIF1A* relative expression (r = 0.703, $P < 0.001$), as shown in Figure 2A. Additionally, the relative expression levels of both genes correlated positively to perifascicular atrophy (r = 0.420, $P = 0.023$ and r = 0.404, $P = 0.003$, for *ANKRD1* and *HIF1A*, respectively), as shown in Figures 2B and 2C.

Nevertheless, both *ANKRD1* and *HIF1A* relative expression levels did not correlate to demographics (age at biopsy muscle procedure, gender and ethnicity), to clinical data (disease duration, muscle strength) and to laboratory features (creatine kinase and aldolase) as shown in Table 1. Moreover, these genes did not correlate to muscle biopsy characteristics as VAS inflammatory infiltration and VAS abnormality.

The *ANKRD1* was localized in the perifascicular area of muscle fibers of DM patients.

DISCUSSION

To our knowledge, this is the first study that addressed *ANKRD1* and *HIF1A* expression in muscle biopsies of DM patients not previously submitted to drug therapy. *ANKRD1* was overexpressed in DM muscle biopsies, and the expression levels were positively correlated to *HIF1A* expression and perifascicular atrophy.

In adult muscles, *ANKRD1* overexpression is generally associated with pathological and stress conditions, as in denervated skeletal muscle,[4,6] muscle pathologies[5,7-9] and inherited myopathies, such as muscular dystrophies,[22,23] congenital myopathies,[24] motor neuron diseases (spinal muscular atrophy)[24] and amyotrophic lateral sclerosis.[25] It is worth noting that all these disorders have atrophy of muscle fibers as a common denominator, as part of the muscle fiber degeneration/regeneration process. Muscle fiber regeneration

Table 1 - Demographic, clinical, laboratory and biopsy features of patients with dermatomyositis, and correlation with ANKRD1 and HIF1A relative expression.

Features	Dermatomyositis (n=29)	ANKRD1 *P* value	HIF1A *P* value
Age at biopsy muscle procedure (years), mean	44.2±18.1	0.100	0.080
Disease duration (months), interquartile	6 (5-12)	0.123	0.236
Gender (female), %	23 (79.3)	1.000	0.173
Ethnicity (white), %	35 (86.2)	0.711	0.711
Muscle strength			
Upper limbs			
Grade III, %	3 (10.4)	0.070	0.155
Grade IV, %	21 (72.4)		
Grade V, %	5 (17.2)		
Lower limbs			
Grade III, %	3 (10.4)	0.183	0.951
Grade IV, %	21 (72.4)		
Grade V, %	5 (17.2)		
Laboratory alterations			
Creatine phosphokinase (IU/L), interquartile	2200 (1805-3335)	0.879	0.517
Aldolase (IU/L), interquartile	43 (17-167)	0.809	0.605
Muscle biopsy			
Number of fascicule / sample, interquartile	10 (4-20)	-	-
Perifascicular atrophy, %	10 (34.5)	-	-
VAS inflammatory infiltration (0-10mm), interquartile	0.8 (0.4-2.0)	0.106	0.156
VAS abnormality (0-10mm), interquartile	0.8 (0.6-1.8)	0.106	0.339

Results are expressed as mean ± standard deviation, median (25th – 75th interquartile) or percentage (%).
ANKRD1: ankyrin repeat domain 1; HIF1A: hypoxia-inducible factor 1 - alpha subunit; VAS: visual analogue score.

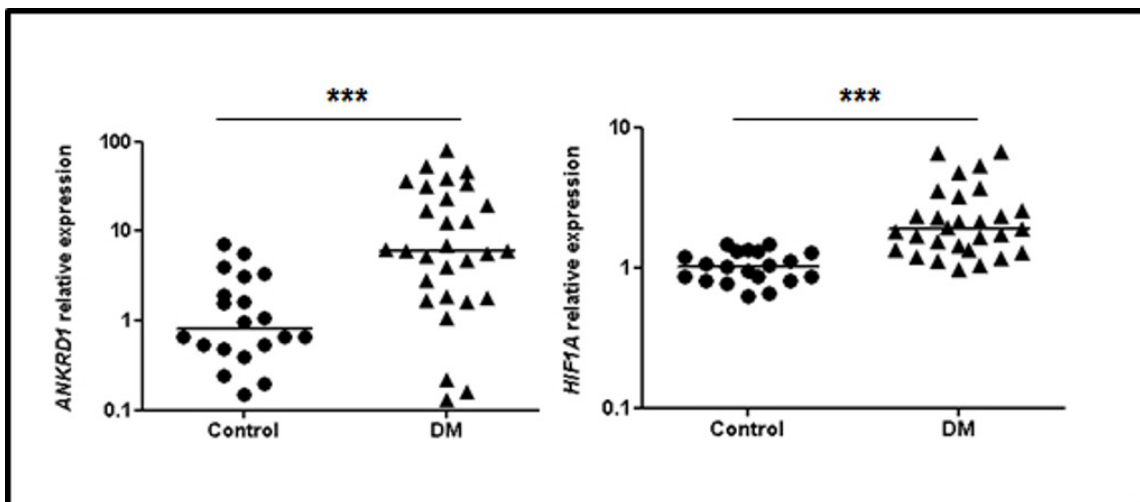

Figure 1. Relative expression profiles of *ANKRD1* and *HIF1A* in dermatomyositis (DM) and control group muscle biopsies. The horizontal bars indicate the median of each control group (*ANKRD1* = 0.815 and *HIF1A* = 0.825) and DM group (*ANKRD1* = 5.950 and *HIF1A* = 1.880). Mann-Whitney test: *** P = 0.0002, for *ANKRD1* and P < 0.0001 for *HIF1A*.

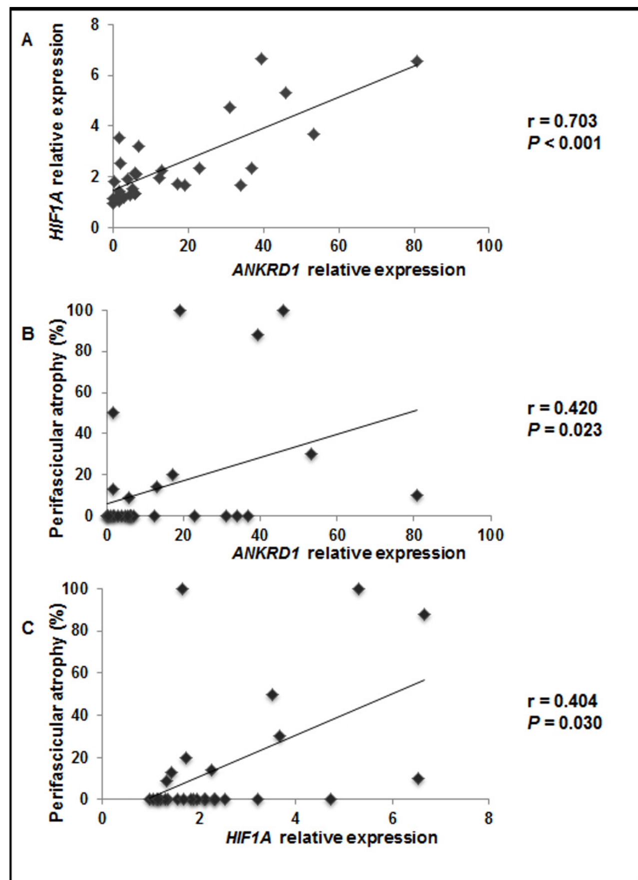

Figure 2. (A) Correlation between *ANKRD1* and *HIF1A* relative expression levels; (B) *ANKRD1* relative expression levels and perifascicular atrophy; and (C) *HIF1A* relative expression levels and perifascicular atrophy for DM cases.

is an important finding in DM muscle biopsy, which may be related to high expression of *ANKDR1*. Upregulation of ANKRD1 has been demonstrated in the early stage of muscle regeneration in animal model of muscle damage,[16] as well as in experimental denervation-induced atrophy and muscular dystrophy models.[9] Therefore, ANKRD1 has been pointed as a general marker of muscle damage. ANKRD1 is also suggested to play an anti-inflammatory role through inhibition of NF-κB transcriptional activity by modulating the balance between physiological and pathological inflammatory responses in skeletal muscle.[26]

ANKRD1 high expression was also demonstrated in skeletal muscle under hypoxic conditions in *in vivo* models.[10] The latter situation hints at a putative link to the adaptation of specific mechanisms in order to maintain adequate oxygenation of critical tissues under hypoxic stress.[10] Moreover, the hypoxic status could activate Hypoxia inducible factors, which act as a master switch to induce expression of several angiogenic factors including vascular endothelial growth factor, nitric oxide synthase, platelet-derived growth factor and others.[27,28]

HIFs are regulated through hydroxylation by oxygen sensitive hydroxylases, notably by factor-inhibiting HIF-1 (FIH-1).[29] HIF hydroxylation directly regulates transcriptional activities of the HIF-induced targets. It should be noted that FIH-1 appears to have high affinity to many substrates containing the ankyrin repeat motif. In fact, FIH-1 hydroxylates well conserved asparagine residues within the ankyrin repeats.[29] The affinity of FIH-1 for many ankyrin containing proteins is higher than its affinity for HIF-1; therefore, oxygen sensitive hydroxylases may directly regulate activity of proteins with ankyrin repeat proteins implicating an active role in the hypoxia response mechanisms.

The atrophic muscle fibers from perifascicular areas correlated with *ANKDR1* expression. Moreover, *ANKRD1* expression correlated significantly to *HIF1A* expression and to the degree of perifascicular atrophy. This perifascicular muscle fiber atrophy is associated to microvascular capillary pathology, including a reduction in the number as well as in the enlargement of some remaining endomysial capillaries.[30] Apparent capillary loss is most prominent within regions of muscle fiber atrophy at the edge of fascicles. Deposition of C5b9 complement on endomysial capillaries has been reported as further evidence that microvasculopathy may play a pathogenic role in DM.[31-33] This condition could promote a hypoxic tissue status that generates a sequence of gene expressions involved in hypoxia, including *HIF1A* and consequently *ANKRD1* gene. Furthermore, in the present study, the immunohistochemical analysis showed that *ANKRD1* and *HIF1A* presented higher expression in muscle fibers in perifascicular area, corroborating our present hypothesis. Nevertheless, *ANKRD1* and *HIF1A* expression did not correlate to any demographic, clinical or laboratory parameters.

SUMMARY

ANKRD1 was overexpressed in muscle biopsies of patients with DM and correlated positively to perifascicular atrophy and *HIF1A* gene expression. Further investigation about *ANKRD1* involvement in myogenesis and angiogenesis mechanisms will better clarify the pathomechanism in DM.

AUTHOR CONTRIBUTION

S K Shinjo: planning, reviewing literature, executing and writing the present article.

S M Oba-Shinjo: planning, reviewing literature and writing the present article.

M Uno: planning, executing and writing the present article.

S K N Marie: planning, reviewing literature and writing the present article.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

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A EXPRESSÃO DO GENE ANKRD1 CORRELACIONA-SE COM O STATUS DE BIÓPSIAS MUSCULARES EM ADULTOS PORTADORES DE DERMATOMIOSITE SEM EXPOSIÇÃO PRÉVIA A TRATAMENTO

OBJETIVOS: *ANKRD1* codifica "ankyrin repeat domain containing protein 1" e tem um papel importante na miogênese e possivelmente também na angiogênese. Microvasculopatia é considerada como um ponto central e uma alteração patológica precoce na dermatomiosite (DM), levando à hipóxia e à atrofia perifascicular muscular. Estas alterações poderiam estimular genes envolvidos na miogênese e angiogênese como *ANKRD1*. Portanto, analisamos a expressão de *ANKRD1* em biópsias musculares de DM e correlacionamos com outros parâmetros de hipóxia e alterações histológicas.

MÉTODOS: O RNA total foi extraído de biópsias de músculos congelados de 29 pacientes com DM. Como grupo controle, foram usadas 20 biópsias de músculo de pacientes adultos com miopatia não-inflamatória. O gene que codifica a subunidade alfa do fator 1 induzido por hipóxia (*HIF1A*) foi analisado para estimar o grau de hipóxia. Os níveis de expressão dos transcritos *ANKRD1* e *HIF1A* foram determinados por PCR quantitativa em tempo real.

RESULTADOS: Níveis aumentados de expressões de *ANKRD1* e *HIF1A* foram observados em DM quando comparados ao grupo controle (P<0,001, ambos os genes). Além disso, *ANKRD1* e *HIF1A* apresentaram coexpressão (r=0,703, P=0,001) e seus níveis de expressão correlacionaram-se também positivamente com atrofia perifascicular (r=0,420, P=0,023 e r=0,404, P=0,030, respectivamente).

CONCLUSÕES: Nossos resultados demonstraram aumento de expressão de *ANKRD1* na DM, que correlacionou com a expressão de *HIF1A* e atrofia perifascicular. Investigações adicionais do envolvimento de *ANKRD1* no mecanismo de miogênese e angiogênese devem ser realizadas.

PALAVRAS-CHAVE: Dermatomiosite, hipóxia, miogênese, atrofia parafascicular, expressão de RNA.

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