Yin Yang 1 Expression and Localisation in Quadriceps Muscle in COPD

Samantha Amanda Natanek, Joanna Riddoch-Contreras, Gemma Sarah Marsh, Nicholas Stephen Hopkinson, William D.-C. Man, John Moxham, Michael Iain Polkey, Paul Robert Kemp

Introduction: Yin Yang 1 (YY1) is a transcriptional repressor that inhibits muscle gene expression and myogenesis. YY1 has not previously been investigated in the skeletal muscle of patients with COPD. The aims of this study were to investigate YY1 expression and localisation in the quadriceps muscle of COPD patients compared to healthy age-matched controls, and to examine the relationship between YY1 expression and localisation and quadriceps muscle fibre cross-sectional area (CSA) in COPD patients. Patients and methods: 15 COPD patients and 8 age-matched controls underwent lung and quadriceps function assessments and a percutaneous quadriceps biopsy. Quadriceps muscle fibre CSA and fibre proportions and YY1 localisation were determined by immunofluorescence. YY1 was immunoprecipitated from muscle and YY1 levels assessed by western blotting. Results: YY1 levels were inversely correlated with type IIx and type I fibre CSA in patients and controls, though YY1 levels were not significantly different between the groups. Nuclear localisation of YY1 was demonstrated in the patients but not in controls. Conclusion: YY1 expression is associated with smaller quadriceps fibre CSA in COPD and nuclear localisation of YY1 was found in muscle of patients but not controls. Regulation of YY1 appears altered in COPD and may be implicated in COPD-related muscle atrophy.

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Expresión y localización del factor de transcripción Yin Yang 1 en el músculo cuádriceps en la enfermedad pulmonar obstructiva crónica

Introducción: El Ying Yang 1 (YY1) es un factor de transcripción represor que inhibe la expresión génica muscular y la miogénesis. Este factor no se ha investigado previamente en el músculo esquelético de pacientes con enfermedad pulmonar obstructiva crónica (EPOC). Los objetivos del presente estudio fueron investigar la expresión de YY1 y su localización en el músculo cuádriceps de pacientes con EPOC, comparado con individuos control sanos, emparejados por edad, y examinar la relación entre la expresión y localización de YY1 en las áreas transversales (AT) de las fibras musculares del cuádriceps en pacientes con EPOC. Pacientes y métodos: Se sometió a 15 pacientes con EPOC y a 8 individuos control, emparejados por edad, a valoraciones de la función pulmonar y del cuádriceps y a una biopsia percutánea de este músculo. Mediante inmunofluorescencia se determinó el AT de las fibras musculares del cuádriceps las proporciones de fibras y localización de YY1. YY1 se inmunoprecipitó a partir del músculo y sus niveles se evaluaron mediante inmunotransferencia.

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In order to maintain muscle mass, regeneration of the skeletal muscles is necessary. However, based on animal models in COPD, there is evidence that skeletal muscle regeneration may deteriorate as a consequence of systemic inflammation, which contributes to the muscle atrophy. We still do not fully understand the molecular mechanisms that may translate into a deterioration of the myogenesis in COPD.

In accordance with the guidelines of the American Thoracic/European Respiratory Society, we determined postbronchodilator spirometry, lung volumes with plethysmography, and carbon monoxide diffusing capacity, while arterial blood gas was examined from an arterialised blood sample obtained from an earlobe. Lean body mass was determined with bioelectric impedance (Bodystat® 1500, Bodystat, United Kingdom), which was corrected for stature to derive the lean body mass index. Physical activity was determined by a triaxial Dynaport® ADL3 accelerometer (McRoberts BV, Netherlands) that the patients used for two days, 12 h each day, during normal activity. The mean locomotion time was calculated as previously described. The strength of the quadriceps (right leg) was evaluated with the maximum voluntary isometric contraction, in supine decubitus, based on the method by Edwards. The percutaneous needle biopsy in the right vastus lateralis was done with local anaesthesia using the Bergstrom technique. The samples for the analysis of ribonucleic acid and proteins were immediately frozen in liquid nitrogen, while the histology samples were introduced in pre-cooled isopentane for 15 s before being frozen in liquid nitrogen then stored at −80 °C.

Analysis of the Muscle Biopsy

There was not enough tissue from each and every individual to complete all the analyses, therefore for each analysis a subgroup of samples was used.

Immunofluorescent Detection of Yin Yang 1

The frozen 10 μm slices from 10 patients and 8 control individuals were set in a solution of 10% formaldehyde, washed in Triton X-100 at 0.1% in a neutralised saline solution with phosphate buffered saline (PBS) and were blocked with 5% bovine serum albumin (BSA) in PBS, before incubation with rabbit anti-YY1 antibody (dilution 1:400, sc-281; Santa Cruz Biotechnology, United States) and murine anti-heavy chain myosin fast contraction antibody (MYSN02, dilution 1:200, Abcam, United Kingdom) in 3% BSA.
in PBS for the entire night at 4 °C. After an incubation of 1 h at room temperature in the dark with secondary antibodies marked with fluorescence (A11008 Invitrogen and A11005 Invitrogen, dilution 1:250 in PBS), the slices were treated with diamino-phenylynide (DAPI). The images (two fields per sample, two cuts per individual) were obtained using a wide-field Zeiss Axiovert microscope, with a 10× lens and Improvision Volocity software. Using a Leica SP2 microscope, we obtained confocal images with a 63× oil immersion lens and they were analysed with LCS Lite software (Leica Microsystems, Germany).

Quantification of the Yin Yang 1 Protein

Y1 is not abundant in the skeletal muscle of adults and, consequently, before immunoblotting, immunoprecipitation was necessary. From nine patients and seven control individuals, for this analysis there were 3–6 mg of protein.

Immunoprecipitation of Yin Yang 1

Between 3 to 6 mg of homogenised protein were incubated in a solution with Nonidet P40, cocktails of protease inhibitor and phosphatase inhibitor (Sigma, Poole Dorset, United Kingdom) in ice for 30 min with 30 μl de Protein G-Sephorose beads. After centrifuging for 2 min (8,000 rpm), the supernatant was incubated for 1 h in ice with 1 μg of rabbit anti-YY1 antibody (sc-281, Santa Cruz Biotechnologies, United States). For each sample, another 30 μl Protein G-Sephorose beads were added and they were re-incubated in ice for 1 h. The samples were centrifuged again and the supernatant was discarded. The beads were washed in Nonidet P40 and boiled with 30 μl of the sample buffer (loading buffer and 2-mercaptoetanol) for 5 min at 100 °C. The process was optimised to guarantee that the supernatant would not have a detectable quantity of YY1 in the Western blot.

Western Blot Analysis

The samples were analysed using electrophoresis in polyacrylamide gel with sodium dodecyl sulphate (SDS/PAGE) (10% gel) and a semidry immunoblotting technique (LKB). After blocking (5% BSA in PBS), the membranes were incubated with mouse anti-YY1 antibody (ab58066, dilution 1:200, Abcam, United Kingdom) in 3% BSA in PBS for the whole night at 4 °C, and immediately followed with an IgG murine antibody bound with horseradish peroxidase (ab 6728, Abcam, United Kingdom, 1:5000 in 3% BSA in PBS) for 1 h at room temperature. The proteins were visualised with Supersignal (Pierce, Rockford, IL, United States), determining the density of the band with a densitometry 1D analysis (AIDA, Raytek, Sheffield, United Kingdom) and was normalised for the quantity of proteins used in the immunoprecipitation.

Determination of the Transversal Area of the Muscle Fibres

From all the subjects, the 10 μm frozen transverse muscle slices were incubated with primary antibodies for type I myosin, type Ila myosin and laminin (A4.840 and N2.261 Developmental Studies Hybridoma Bank, University of Iowa, Unites States, and L-9393 Sigma, Zwijndrecht, Netherlands, respectively) followed by secondary antibodies marked with fluorescence (A-21121, A-21426 and A-11069, Invitrogen). The epifluorescence signal was registered using a Texas Red excitation filter (540–580 nm) for type I myosin, an FITC excitation filter (465–495 nm) for type Ila myosin and a UV DAPI excitation filter (340–380 nm) for the laminin using a Nikon Eclipse 800 microscope. The fibres were classified into type I, Ila, IIX (without staining) and hybrid I/Ila (dual staining) and we used the laminin edge of the fibres to calculate the CSA of each fibre.

| Table 1: Clinical Characteristics of the Patients and Control Subjects. |
|----------------|----------------|
| **COPD Patients (n=15)** | **Control Subjects (n=8)** |
| Age, years | 69 (63.75) | 69 (65.76) |
| Sex | 53% males | 50% males |
| Tobacco habit | 2 current and 13 ex-smokers | 8 ex-smokers and 2 non-smokers |
| Smoking history, pack/year | 45 (30.75)\(^a\) | 7 (1.14) |
| FEV\(_1\), l | 0.86 (0.59, 1.05)\(^a\) | 2.92 (2.53, 2.98) |
| FEV\(_1\), % reference value | 33 (22, 54)\(^a\) | 109 (105, 112) |
| TLC, % reference value | 36 (25, 62)\(^a\) | 89 (81,99) |
| PaO\(_2\), kPa | 9.8 (8.0, 10.4) | 10.4 (8.7, 11.4) |
| Body mass index, kg/m\(^2\) | 22.9 (20.7, 26.2) | 24.8 (21.0, 26.7) |
| Lean body mass index, kg/m\(^2\) | 16.2 (14.3, 18.6) | 15.8 (14.5, 19.5) |
| MVE of quadriceps, kg | 22 (17, 34)\(^b\) | 34 (28, 42) |
| Locomotion time, min/12 h | 57 (21, 75) | 61 (56, 159) |

The values are means (25th percentile and 75th percentile).

\(^a\) P<0.0001 for the group comparisons (analysed with the Mann–Whitney U test).

\(^b\) P<.03 for the group comparisons (analysed with the Mann–Whitney U test).

MVC: maximal voluntary contraction; COPD: chronic obstructive pulmonary disease; PaO\(_2\): partial pressure of oxygen; TLC: carbon monoxide diffusing capacity; FEV\(_1\): forced expiratory volume in one second.

Quantification of the Levels of Tumour Necrosis Factor \(\alpha\) mRNA

The TNF-\(\alpha\) mRNA transcripts were determined by a chain reaction of the quantitative polymerase, in real time, using SYBR\textsuperscript{\textregistered} Green PCR Master Mix (Applied Biosystems) in a 7900HT Fast Real-Time PCR System (Applied Biosystems). The transcripts normalised to a geNorm factor derived from two internal genes, acidic ribosomal phosphoprotein (RLPPO) and beta-2 microglobulin (\(\beta_2\)M), as previously reported.\(^{24}\) For each individual, we analysed an average of 207 fibres, with a minimum of 103 fibres.

Statistical Analysis

The data did not have normal distribution (according to the histogram and the test of symmetry), so they are reported as averages (25th percentile and 75th percentile). The group differences in the continuous variables were analysed with the Mann–Whitney U-test, while the Fisher’s exact test was used to test the group differences in the categorical variables. Spearman’s rank correlation coefficient (\(\rho\)) was calculated to determine the relationship among the variables (Statview 1.0, Abacus Instruments). To define the statistical significance, a two-tailed \(P\) value ≤.05 was used.

Results

Table 1, shows the physiological data of the patients and controls. As was expected, the patients presented a deterioration in lung function and a decrease in the strength of the quadriceps, in comparison with the control individuals [maximal voluntary contraction 22 (17.34) kg compared with 34 (28.42) kg in control subjects, \(P=.03\)].
Table 2
Levels of YY1, HDAC 5, and HDAC 5 Bound With YY1 and Mean CSA of the Type I, I/IIa, IIa and IIx Fibres in the Quadriceps of Patients With COPD and Control Individuals.

<table>
<thead>
<tr>
<th></th>
<th>COPD Patients (n=15)</th>
<th>Control Subjects (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YY1, AU/mg protein</td>
<td>3.25 (1.95, 7.17) (n=9)</td>
<td>3.84 (2.18, 6.01) (n=7)</td>
</tr>
<tr>
<td>Levels of mRNA-TNF-α, AU</td>
<td>0.08 (0.03, 0.11) (n=15)</td>
<td>0.04 (0.03, 0.05) (n=8)</td>
</tr>
<tr>
<td>CSA type I fibre, µm²</td>
<td>5352 (4151, 6783) (n=15)</td>
<td>5644 (4111, 7411) (n=8)</td>
</tr>
<tr>
<td>CSA type I/IIa fibre, µm²</td>
<td>4971 (3292, 7306) (n=15)</td>
<td>5754 (5006, 5956) (n=3)</td>
</tr>
<tr>
<td>CSA type Ila fibre, µm²</td>
<td>4221 (2996, 5170) (n=15)</td>
<td>3314 (3002, 5408) (n=15)</td>
</tr>
<tr>
<td>CSA type Ix fibre, µm²</td>
<td>3439 (2348, 3739)² (n=14)</td>
<td>5015 (3618, 6303) (n=8)</td>
</tr>
<tr>
<td>Percentage of type I fibres</td>
<td>34 (11, 38) (n=15)</td>
<td>52 (47, 63) (n=8)</td>
</tr>
<tr>
<td>Percentage of type I/IIa fibres</td>
<td>3 (1, 6) (n=15)</td>
<td>0 (0, 4) (n=8)</td>
</tr>
<tr>
<td>Percentage of type Ila fibres</td>
<td>60 (52, 70) (n=15)</td>
<td>40 (35, 46) (n=8)</td>
</tr>
<tr>
<td>Percentage of type Ix fibres</td>
<td>3 (1, 16)² (n=15)</td>
<td>1 (0, 4) (n=8)</td>
</tr>
</tbody>
</table>

The values are means (25th percentile, 75th percentile). The percentages of fibres in each individual reach 100% (the sum of the means in each group does not necessarily equal 100% due to the variance around the mean). The levels for mRNA normalise for the transcripts of RPLPO (internal gene).

² p < 0.005 for the group comparisons (analysed with the Mann–Whitney U test).

³ p < 0.05 for the group comparisons (analysed with the Mann–Whitney U test).

CSA: cross-sectional area; HDAC: histone deacetylase; RPLPO: acidic ribosomal phosphoprotein; TNF-α: tumour necrosis factor-alpha; AU: arbitrary units; YY1: Yin Yang 1.

The CSA in the type Ila fibres diminished and the quotient type I fibres: type II fibres decreased in patients with COPD in comparison with control individuals.

In the patients, a significantly smaller CSA was detected in the type Ila fibres than in control individuals [3.439 (2.34, 3.739) µm² compared with 4.628 (4.087, 6.303) µm² respectively, P<0.05], but there was no significant difference in the CSA of the other types of fibres between groups, as had been previously reported.26 In patients, we detected a significantly lower proportion of type I fibres and a greater proportion of type Ila fibres than in control individuals [34 (14, 39)% compared with 52 (47, 63)% and 60 (49, 70)% compared with 40 (35, 46)% respectively], as previously described27 (Table 2).

The levels of YY1 in the quadriceps were associated with a decrease of the CSA in the muscle fibres in COPD.

The quantity of YY1 protein in the quadriceps was not significantly different in patients compared with the control subjects (Fig. 1, Table 2). In patients and controls, the levels of YY1 protein inversely correlated with the CSA of the type Ila fibres (ρ=-0.83, P<0.001); likewise it was true with the CSA of the type I fibres (ρ=-0.59, P=0.017), and there was also an observed tendency towards a negative correlation with the CSA of the type Ila fibres (ρ=-0.43, P=0.09) (Fig. 2). Only in the group of patients did we identify a correlation between the levels of YY1 and in the muscle the CSA of the type Ila fibres (ρ=-0.88, P=0.002). The muscle levels of YY1 did not correlate with the forced expiratory volume in one second or the CO₂ diffusing capacity (TDLCO) as a percentage of the reference value, maximal voluntary contraction of the quadriceps or the lean mass index in patients and control subjects (P=0.69, 0.43, 0.94 and 0.21, respectively) or correlation was only identified in the patient group.

The muscle TNF-α mRNA levels were higher in patients than in control subjects (0.08 [0.03, 0.1] arbitrary units [AU] compared with 0.04 [0.03, 0.05] AU, P=0.05). The TNF-α mRNA transcripts and the levels of YY1 did not correlate in patients and control subjects (P=0.38) or in the patients alone.

The muscle CSA of the type I fibres was only detected in 50% of the patients compared with the control individuals.
Fig. 3. Image from a wide-field microscope of a longitudinal slice of the quadriceps muscle of a healthy control subject demonstrating the localisation of YY1 regarding the muscle nuclei by means of immunohistochemistry. The muscle was stained for YY1 (green), the heavy chain of the fast myosin (red) and the nuclei using 4',6-diamidino-2-phenylindole (blue). The YY1 staining is observed along the sarcomeres, not co-localised with the myosin, and the absence of nuclei is also observed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Nuclear Localisation of Yin Yang 1 in the Muscle of a Patient Subgroup With Chronic Obstructive Pulmonary Disease but not in Control Individuals

YY1 was absent from the nucleus but present in the cytoplasm in the quadriceps of the healthy control subjects (Figs. 3 and 4C, D, G, H). YY1 in the cytoplasm aligned with the sarcomeres, but it did not co-localise with myosin (Fig. 3).

YY1 staining was demonstrated in the nuclear region in none of the control individuals but in 5 of the 10 patients. This difference between groups was statistically significant (Fisher’s exact test, \(P=0.04\)). Compared with 6 of the 8 control subjects, only in 2 of the 10 patients was there a demonstrated exclusively cytoplasmic YY1 distribution that, once again, was statistically significant (Fisher’s exact test, \(P=0.05\)). No significant difference was identified between patients with or without nuclear YY1 localisation regarding muscle or lung function, quadriceps fibre CSA or YY1 protein levels.

Discussion

We have made a new finding: that the YY1 transcription factor, a repressor of muscle-specific gene expression and myogenesis, is expressed in a proportion inverse to CSA of the type IIx and type I fibres in the quadriceps muscle of patients with COPD. In a significant proportion of these patients, the nuclear localisation of the factor was evident, which contrasts with the distribution in the muscle of healthy adults.

Critique of the method

Naturally, the present study presents limitations. First of all, the size of the sample was small and the associations detected had to stand out; therefore it is possible that no other associations or group differences were identified due to the lack of power. The control subjects included in the present study were characterised by a relatively low lean mass index and a wide variance in their degree of physical activity, for which there is no clear explanation, which increases the threshold for the statistical significance of the group differences. Although the study of patients in stage II, III, and IV of the GOLD initiative should have made it easier to find correlations between the muscle and lung function and the YY1 levels (because the data was not expected to gather), the exclusive use of patients in stage IV could have maximised the probability of finding a significant difference in YY1 expression between patients and control subjects. Second, the observational design does not allow for the extraction of conclusions about the effect of the differences in YY1 on muscle structure and function, which merely highlights an association between the increase in expression of the factor and the smaller size of the fibres and between the nuclear localisation of the factor and the presence of COPD and less strength in the quadriceps muscle than in control individuals. Lastly, we used the
localisation and expression of the factor as an indirect variable of the activity because YY1 is activated by transport from the cytoplasm to the nucleus. Our findings could have been corroborated by the quantification of YY1 bound to DNA (immunoprecipitation of chromatin, a technique that has not been previously published as being used in human muscle) or the quantification of YY1 able to bind with DNA (for example, by means of electrophoretic analysis of the change in mobility).

It could also be argued that the YY1 factor detected in the nuclei is not present in the periphery of the myofibrils, but is instead in the latent, satellite cells. However, we consider that this is improbable as the satellite cells only represent 2%-5% of the muscle nuclei. Even supposing a massive increase in the population of satellite cells in the tissue of the patients, the number of nuclei that stained for the factor considerably surpasses the number that could be justified by the satellite cells. Furthermore, in the samples of this present study, the absence of centralised nuclei suggests a limited activation of the satellite cells in the period of time when the samples were taken.

**Significance of the Findings**

The implication of a powerful inverse correlation between the levels of the YY1 factor and the size of the fibres is that YY1 could participate in the mechanism of fibre atrophy, particularly as the relationship is stronger with the CSA of the type Ix fibres and these fibres seem to be the type that atrophy the most in COPD. However, this hypothesis requires additional research, for example, examining the effect in the skeletal muscle of adult knockout or knockdown mice with emphysema induced by tobacco smoke or other means.

The finding that patients with a nuclear localisation of the factor do not necessarily have higher levels can be explained by the fact that its localisation and expression can be regulated independently. It is possible that the patients with a greater expression of YY1 and a smaller size of the fibres are characterised by greater levels of NF-kB activation in the muscle, while in others a greater reserve of depolymerised actin would give rise to a nuclear accumulation of the factor without an increase in its expression. However, we did not find a correlation between the local values of TNF-α and YY1 in the muscle of the patients, which supports the suggestion that the levels of the factor are mainly determined by local TNF-α that would activate the NF-kB factor.

In conclusion, we report the new finding that YY1, transcription factor for the repression of the specific gene expression of muscles and myogenesis, is expressed associated with a decrease in the CSA of type Ix and type I fibres of the quadriceps muscle in COPD. YY1 is also localised in the nuclei of the quadriceps of COPD patients in comparison with healthy control subjects, paired by age. We speculate that the greater activity of YY1 could be implicated in the deterioration of skeletal muscle regeneration and the atrophy of the muscle fibres in COPD.

**Conflict of Interest**

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**References**


