Colocalization of Transactivation-Responsive DNA-Binding Protein 43 and Huntingtin in Inclusions of Huntington Disease

Claudia Schwab, PhD, Tetsuaki Arai, MD, PhD, Masato Hasegawa, PhD, Sheng Yu, and Patrick L. McGeer, MD, PhD

Abstract

Transactivation-responsive DNA-binding protein 43 (TDP-43) is a component of pathological inclusions in amyotrophic lateral sclerosis and several forms of sporadic and familial frontotemporal lobar degeneration. Transactivation-responsive DNA-binding protein 43–immunostained inclusions have also been found in other neurodegenerative disorders including Alzheimer disease, dementia with Lewy bodies, and parkinsonism dementia complex of Guam. Here, we analyzed the occurrence of TDP-43 immunostaining in Huntington disease, which is characterized by inclusions containing mutated huntingtin. In all Huntington disease cases studied, TDP-43 was frequently colocalized with huntingtin in dystrophic neurites and various intracellular inclusions, but not in intranuclear inclusions; the latter were only stained with huntingtin and anti-ubiquitin antibodies. Two phosphorylation-dependent TDP-43 antibodies proved to be superior for detecting pathological inclusions because they did not stain nonphosphorylated TDP-43 in normal nuclei; staining of normal nuclei with phosphorylation-independent antibodies obscured the inclusions. Our results further add to the hypothesis that TDP-43 may be involved in the pathology of a variety of neurodegenerative disorders.

Key Words: Huntingtonin, Intracellular inclusions, Phosphorylation-dependent TDP-43 antibodies, TDP-43, Ubiquitin.

INTRODUCTION

Transactivation-responsive DNA-binding protein 43 (TDP-43) has recently been identified as a component of ubiquitin-positive inclusions in several forms of sporadic and familial frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS) (1, 2). Transactivation-responsive DNA-binding protein 43 is ubiquitously expressed and is involved in regulating transcription and alternative splicing. It was originally described as a suppressor of human immunodeficiency virus 1 gene expression (3) and as a splicing regulator of the cystic fibrosis transmembrane conductance regulator (4).

In ALS and FTLD, TDP-43 inclusions occur as neuronal intranuclear inclusions (NIIs), neuronal cytoplasmic inclusions, and dystrophic neurites (DNs) in characteristic patterns in different subtypes of FTLD (2, 5–8). Transactivation-responsive DNA-binding protein 43 is normally a nuclear protein, but its level is reduced or even undetectable in the nucleus in neurons when pathological inclusions are present (2, 9).

Transactivation-responsive DNA-binding protein 43 has recently been found to be colocalized with inclusions in neurodegenerative conditions other than FTLD and ALS, suggesting that the protein may be involved in the pathogenesis of a variety of disorders. Several reports described TDP-43–immunopositive inclusions in various proportions of cases with Alzheimer disease (20%–33%) (10, 11), hippocampal sclerosis (70%) (10), parkinsonism dementia complex of Guam (12, 13), diffuse Lewy body disease, Parkinson disease (11, 14), and corticobasal degeneration (15); in some reports, these inclusions were not seen in Parkinson disease, Pick disease, progressive supranuclear palsy, corticobasal degeneration, or frontotemporal dementia with tau mutations (FTDP-17) (11).

In the present study, we investigated colocalization of TDP-43 with the huntingtin (htt)-containing inclusions of Huntington disease (HD). Huntington disease is a hereditary polyglutamine disorder caused by expansion of the triplet repeat region of the htt gene. It has been shown that mutant htt is cleaved by caspases after which it undergoes nuclear translocation (16, 17). Huntingtonin-immunostained inclusions are found in the nuclei and cytoplasm of neurons in the form of htt NIIs and htt DNs (18–22).

MATERIALS AND METHODS

Ten cases of HD, 1 case of ALS, and 2 elderly control cases were selected for study from our brain bank at the University of British Columbia (Table 1).

Immunohistochemistry was performed on CNS tissue samples using a panel of antibodies against htt, ubiquitin, phosphorylated neurofilament H, and TDP-43. The antibody sources and dilutions used are shown in Table 2. Except for 2 antibodies to phosphorylated TDP-43, they were obtained from commercial sources. The phosphorylated TDP-43 antibodies were raised against phosphorylated sequences of
human TDP-43 (23) and have the advantage of not recognizing normal TDP-43, which is not phosphorylated.

Tissues had been fixed in 4% paraformaldehyde, and after 3 to 4 days, transferred to a 15% buffered sucrose maintenance solution. For immunohistochemistry, 30-Km-thick sections of selected areas were cut on a freezing microtome (American Optical Corporation, Buffalo, NY). For light microscope immunostaining, the sections were treated for 30 minutes with 0.5% hydrogen peroxide solution in 0.01 mol/L PBS, pH 7.4, containing 0.3% Triton X-100 (PBS-T), transferred into 5% skim milk in PBS-T for 30 minutes, and incubated for 72 hours at 4°C or overnight at room temperature with one of the primary antibodies.

Sections were next treated with the appropriate biotinylated secondary antibodies (DAKO, Mississauga, Toronto, Ontario, Canada; 1:2000) for 2 hours at room temperature, followed by incubation in avidin-biotinylated horseradish peroxidase complex (DAKO; 1:10000) for 1 hour at room temperature. Peroxidase labeling was visualized by incubation in 0.01% 3,3-diaminobenzidine (Sigma, Oakville, Ontario) containing 1% nickel ammonium sulfate (Fisher Scientific, Ottawa, Ontario, Canada), 5 mmol/L imidazole (BDH Laboratory Supplies, Poole, United Kingdom), and 0.001% hydrogen peroxide in 0.05 mol/L Tris-HCl buffer, pH 7.6. When a dark purple/black color developed, sections were washed, mounted on glass slides, air-dried, and coverslipped with Entellan (EMD Biosciences, Gibbstown, NJ). Some sections were counterstained with Neutral Red (BDH Laboratory Supplies) before coverslipping. Antigen retrieval for staining with the monoclonal Abnova TDP-43 antibody was carried out by incubating the sections in boiling PBS for 5 minutes before all other steps to improve the staining of inclusions.

### TABLE 1. Case Material*

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis</th>
<th>Age, years/Sex</th>
<th>Postmortem Interval, hours</th>
<th>Age at Onset/Duration of Disease, years</th>
<th>Brain or Spinal Cord Region</th>
<th>Density of Cortical htt Inclusions†</th>
<th>htt/TDP-43 Double Labeling‡</th>
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</thead>
<tbody>
<tr>
<td>HD1</td>
<td>HD</td>
<td>55/M</td>
<td>3</td>
<td>41/14</td>
<td>IF</td>
<td>Moderate</td>
<td>++</td>
</tr>
<tr>
<td>HD2</td>
<td>HD</td>
<td>59/F</td>
<td>4</td>
<td>NA</td>
<td>IF</td>
<td>Low</td>
<td>+</td>
</tr>
<tr>
<td>HD3</td>
<td>HD</td>
<td>62/M</td>
<td>4.5</td>
<td>NA</td>
<td>PC</td>
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<td>+</td>
</tr>
<tr>
<td>HD4</td>
<td>HD</td>
<td>53/M</td>
<td>3.5</td>
<td>46/7</td>
<td>ST</td>
<td>Low</td>
<td>+</td>
</tr>
<tr>
<td>HD5</td>
<td>HD</td>
<td>49/M</td>
<td>8.5</td>
<td>43/6</td>
<td>MT</td>
<td>Moderate</td>
<td>++</td>
</tr>
<tr>
<td>HD6</td>
<td>HD</td>
<td>56/M</td>
<td>11.5</td>
<td>NA</td>
<td>MT</td>
<td>High</td>
<td>++</td>
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<tr>
<td>HD7</td>
<td>HD</td>
<td>57/F</td>
<td>4</td>
<td>36/21</td>
<td>MT</td>
<td>Low</td>
<td>++</td>
</tr>
<tr>
<td>HD8</td>
<td>HD</td>
<td>43/M</td>
<td>3</td>
<td>30/13</td>
<td>SF, C/P</td>
<td>Moderate</td>
<td>+</td>
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<tr>
<td>HD9</td>
<td>HD</td>
<td>76/F</td>
<td>8</td>
<td>NA</td>
<td>SF, C/P</td>
<td>Moderate</td>
<td>+++</td>
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<tr>
<td>HD10</td>
<td>HD</td>
<td>68/M</td>
<td>32</td>
<td>46/22</td>
<td>Moderate</td>
<td>+</td>
<td></td>
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<tr>
<td>ALS</td>
<td>ALS</td>
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<td>NA</td>
<td>SC</td>
<td>na</td>
<td>na</td>
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<tr>
<td>CO1</td>
<td>Control</td>
<td>72/M</td>
<td>4.5</td>
<td>NA</td>
<td>MF</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>CO2</td>
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<td>4</td>
<td>NA</td>
<td>MF</td>
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<td>na</td>
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</table>

*ALS, amyotrophic lateral sclerosis; C/P, caudate/putamen; F, female; HD, Huntington disease; htf, huntingtin; IF, inferior frontal cortex; M, male; MF, midfrontal cortex; MT, midtemporal cortex; NA, data not available; PC, precentral cortex; SC, spinal cord; ST, superior frontal cortex; TDP-43, transactivation-responsive DNA-binding protein 43.
†See Materials and Methods section.
‡Percentage of htt/TDP-43 double labeled inclusions (of total htt inclusions): (+) 30% to 40%, (++) 41% to 60%, and (+++) 61% to 75%.

### TABLE 2. Antibody Sources and Dilutions*

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Catalogue No.</th>
<th>Antigen/Specificity</th>
<th>Dilutions (3,3-Diaminobenzidine Immunohistochemistry/Fluorescence)</th>
</tr>
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<tbody>
<tr>
<td>Huntingtin (mEM48)</td>
<td>Chemicon, Temecula, CA</td>
<td>MAB5374</td>
<td>Human huntingtin</td>
<td>1:400/1:100</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>DAKO, Mississauga, Toronto, Ontario, Canada</td>
<td>Z0458</td>
<td>Ubiquitin from cow erythrocytes</td>
<td>1:1000/1:200</td>
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<tr>
<td>SMI31</td>
<td>Covance/Cedarlane, Burlington, Ontario, Canada</td>
<td>SMI31-R</td>
<td>Phosphorylated neurofilament H</td>
<td>ND/1:2000</td>
</tr>
<tr>
<td>TDP-43</td>
<td>ProteinTech, Chicago, IL</td>
<td>10782-2-AP</td>
<td>Recombinant TDP-43</td>
<td>1:300/1:100</td>
</tr>
<tr>
<td></td>
<td>Abnova, Taipei, Taiwan</td>
<td>H00023435-A01</td>
<td>Full-length recombinant TDP-43 with GST tag (1-261 AA)</td>
<td>1:300+ND</td>
</tr>
<tr>
<td>TDP-43 ps403/04</td>
<td>Dr M. Hasegawa‡</td>
<td>NA</td>
<td>TDP-43 (398–408, ps403/404)</td>
<td>1:2000/1:100</td>
</tr>
<tr>
<td>TDP-43 ps409/10</td>
<td>Dr M. Hasegawa</td>
<td>NA</td>
<td>TDP-43 (405–414, ps409/410)</td>
<td>1:2000/1:100</td>
</tr>
</tbody>
</table>

*NA, not applicable; ND, not done; TDP-43, transactivation-responsive DNA-binding protein 43.
†Antigen retrieval used for immunohistochemistry.
‡Tokyo Institute of Psychiatry, Tokyo, Japan.
For double immunofluorescence staining, sections were incubated in 5% skim milk in PBS-T for 30 minutes and then incubated for 72 hours at 4°C or overnight at room temperature with a combination of 2 primary antibodies. These combinations were EM48/TDP-43 (pS403/404), EM48/TDP-43 (pS409/410), EM48/TDP-43 (ProteinTech, Chicago, IL), and SM131/TDP-43 (pS403/404) (Table 2). Sections were next incubated with a mixture of fluorophore-labeled secondary antibodies (Alexa Fluor 488 goat-anti-mouse and Alexa Fluor 546 goat-anti-rabbit, Invitrogen, Burlington, Ontario, Canada; 1:500) in the dark, counterstained with Hoechst 33258 (Invitrogen), and mounted on glass slides. To reduce lipofuscin autofluorescence, sections were next treated with a solution of 0.3% Sudan Black B (Gurr Ltd, London, United Kingdom) in 70% ethanol for 7 minutes and washed in PBS 8 times (24). The sections were then air-dried and coveredslipped with Prolong Gold (Invitrogen). The staining pattern for each individual antibody after the double fluorescence staining was identical to the pattern observed in single immunohistochemical staining experiments. Controls for immunostaining were performed by omitting the primary antibodies. No staining was observed in these controls. The density of htt (EM48)-labeled inclusions was assessed quantitatively. The proportion of htt/TDP-43 double-stained inclusions was evaluated semiquantitatively by counting the number of inclusions labeled by EM48 and by TDP-43 antibodies in at least 4 occular fields at 40× magnification.

Confocal images were captured with a spindisk confocal microscope (inverted Olympus widefield microscope with Carv Spindisk, Olympus, Center Valley, PA) at 60× objective magnification. Fluorescent images were colocalized with ImagePro software (Improvement Inc, Waltham, MA). Other images were acquired using a conventional fluorescence microscope (Olympus BX51) and a digital Olympus DP71 camera (Olympus, Center Valley, PA).

RESULTS

Immunohistochemistry with the anti-htt antibody (EM48) revealed several forms of inclusions in cortical areas and to a lesser extent in the basal ganglia of the HD cases. The results are in agreement with earlier reports (18–20). The most abundant inclusions were round, ovoid, or ellipsoid neuropil aggregates of various sizes ranging from approximately 1 to 15 μm in diameter (Fig. 1A–C). Most of these aggregates were observed in the lower layers of the cortex and in the adjacent white matter. Fewer inclusions were also found in the striatum. Cortical sections were therefore selected for colocalization staining. Counterstaining with neutral red revealed that most of the aggregates were not localized inside perikarya or in nuclei; similar inclusions have been referred to as neuropil inclusions (NIs) (19). Some of the NIs had tailed processes or were spindle-shaped, as previously described (18). Small intranuclear inclusions were also present predominantly in the upper cortex layers (Fig. 1C, arrow), and threadlike stained structures were observed in most of the cases. No EM48 staining was detected in the control cases.

Immunostaining with the anti-ubiquitin antibody revealed inclusions of identical shape, distribution pattern, and frequency as demonstrated with the anti-htt antibody EM48 (Fig. 1D–G). It is well established that most htt inclusions in HD are ubiquitinated (19, 25).

In the ALS case, the pTDP-43 (pS403/404) and pTDP-43 (pS409/410) antibodies stained skeinlike and spherical inclusions in anterior horn neurons of the spinal cord as reported by Hasegawa and colleagues (23) (Fig. 1H–J).

In all of the HD cases, all TDP-43 antibodies labeled inclusions similar in shape and distribution to the htt and ubiquitin-immunoreactive inclusions. Various sizes of round, oval, and ellipsoid inclusions were present in the neuropil of lower cortex layers and white matter (NI). In addition, long DNs and thinner neuropil threads were stained with the phosphorylation-dependent TDP-43 antibodies. These NIs and DNs were also stained with the 2 phosphorylation-independent antibodies (Abnova and ProteinTech, Fig. 1K–O) but less frequently than by the 2 phosphorylation-dependent TDP-43 antibodies (pS403/404 and pS409/410, Fig. 1P–U). The staining pattern observed with pTDP-43 (pS403/404) and pTDP-43 (pS409/410) was similar to that with the 2 commercial TDP-43 antibodies with the exception that nuclei, which were strongly stained by the ProteinTech and Abnova antibodies (Fig. 1K–O), remained unstained with the phosphorylation-dependent TDP-43 antibodies. The Abnova TDP-43 monoclonal antibody required antigen retrieval to visualize inclusions and DNs. In contrast to staining with EM48 and anti-ubiquitin antibodies, the TDP-43 antibodies only very rarely stained intranuclear inclusions. Lentiform (“cat’s eye–shaped”) intranuclear inclusions, typical for a particular subtype of frontotemporal dementia (i.e. subtype 4 [6, 7] or subtype 1 [5]), were not found in the HD cases. In 1 HD case, there were 2 neurons with skeinlike inclusions in the midfrontal cortex using the monoclonal (Abnova) TDP-43 antibody (Fig. 1O).

Double Immunofluorescence Staining

In double-immunofluorescence–stained cortical HD sections, TDP-43 and EM48 appeared to be colocalized frequently in NIs, DNs, and neuropil threads (Fig. 2A–L). In all HD cases, the frequency of inclusions stained with the EM48 antibody exceeded that of inclusions stained with TDP-43 (pS403/404) or TDP-43 (pS409/410). The percentage of TDP-43/htt (EM48) double-stained inclusions varied between 30% and 70% of htt (EM48)-labeled inclusions between the individual cases (Table 1). In addition to many inclusions that were double labeled for both TDP-43 and EM48, some inclusions were labeled with EM48 alone (Fig. 2E, H). Inclusions that were single labeled using TDP-43 antibodies were very rare and were usually large elongated or round DNs. Colocalization of EM48 and TDP-43 was confirmed by examination with a confocal microscope. In some inclusions, EM48 immunostaining appeared to be peripheral around a core of TDP-43 immunofluorescence; in others, both EM48 and TDP-43 antibodies labeled the inclusions throughout. Colocalization was observed with all 3 polyclonal TDP-43 antibodies, but similar to the light microscopic staining, pTDP-43 (pS403/404) and pTDP-43 (pS409/410) labeled more inclusions compared with the ProteinTech antibody. The phospho-TDP-43–labeled inclusions were not colocalized with or in proximity to

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Hoechst-labeled nuclei. This suggests that the inclusions were in DNs and not in perikarya or nuclei (Fig. 2C, G, K).

Double-fluorescence staining with TDP-43 and SMI31 antibodies demonstrated that some of the TDP-43 positive spherical inclusions were localized in DNs stained with anti-neurofilament SMI31 antibody (Fig. 2M, T).

DISCUSSION

The demonstration of TDP-43 as a component of inclusions in sporadic and familial forms of ALS and FTLD has led to the hypothesis that these disorders share a common etiology as “TDP-43 proteinopathies.” Transactivation-responsive DNA-binding protein 43 antibodies label inclusions in FTLD patients who carry mutations in the progranulin and valosin-containing protein genes and in chromosome 9-linked familial forms, but they do not label structures in patients with superoxide dismutase 1 mutations (6, 26, 27). Transactivation-responsive DNA-binding protein 43 has been found in inclusions in other neurodegenerative disorders such as hippocampal sclerosis, Alzheimer disease, Parkinson disease, diffuse Lewy body disease, parkinsonism dementia complex of Guam, and corticobasal degeneration although colocalization was described only in some cases (10–15). Both TDP-43 and tau colocalize in the same neurons in...
tauopathies but they frequently label different inclusions in the same cell with little overlap. Transactivation-responsive DNA-binding protein 43 labeling was reported to show more overlap with α-synuclein staining in diffuse Lewy body disease and Parkinson disease (11). Nevertheless, because TDP-43-containing inclusions are not limited to FTD and ALS, TDP-43 may play a more general role in neurodegeneration than previously assumed.

In this present study, we describe the colocalization of TDP-43 with the htt inclusions of HD. Antibodies against the 2

![FIGURE 2. Confocal imaging of double-immunostained sections of Huntington disease cortex. (A–L) Double staining for huntingtin (htt) green; htt/EM48) and transactivation-responsive DNA-binding protein 43 (TDP-43) red; [A–D] pS403/404, [E–L] pS409/410) counterstained with Hoechst 33258 (blue). Most inclusions were double labeled for both TDP-43 and htt, with htt staining the periphery and TDP-43 the center of the same inclusion. Some inclusions are labeled with htt only ([E, H] arrow). Asterisks in (C, G, and K) indicate that TDP-43/htt double labeled inclusions are distinct from the Hoechst-stained nuclei. (M–T) Sections show 2 examples of localization of TDP-43 and SMI31, a marker for phosphorylated neurofilament, in the same neurons. Note that the TDP-43 inclusions were contained in swollen sections of the neurites.](image-url)
proteins labeled large proportions of HD DNs and cytoplasmic inclusions, but intranuclear inclusions were only immunostained with the anti-htt and anti-ubiquitin antibodies. The colocalization was demonstrated with 3 different polyclonal antibodies against TDP-43, 2 of them directed against phosphorylated epitopes. The monoclonal TDP-43 antibody we used stained similar inclusions in single-label experiments. The observed direct overlap in localization of TDP-43 and htt differs from that observed in tauopathies such as Alzheimer disease, parkinsonism dementia complex of Guam, and Lewy body diseases (10–15). The types of inclusions found in HD are similar to the types found in tauopathies and Lewy body diseases, where neuronal cytoplasmic inclusions and DNIs have been described, but NIIs were not found (14). Although in Lewy body disease TDP-43 inclusions were described more frequently in the superficial cortical layers (14), and Lewy bodies occur throughout the cortex in advanced Lewy body disease (28), we found that the TDP-43 inclusions were more numerous in the lower layers of the HD cortex, where htt inclusions predominate. Although intranuclear htt inclusions are present in HD (NII), TDP-43 was not found in this type of inclusion, as was demonstrated with the Hoehst 33258 counterstaining (Fig. 2C, G, K).

The TDP-43–stained inclusions in HD were most evident using the phosphorylation-dependent antibodies (23) because they were not obscured by staining of the “normal” intranuclear nonphosphorylated protein. This indicates that the phosphorylation-dependent antibodies are excellent tools to study pathological changes involving TDP-43. Posttranslational modification is a common motif in pathological protein accumulation in many neurodegenerative disorders. This process can involve phosphorylation (tau, α-synuclein, TDP-43) (29–32), cleavage (htt, α-synuclein, TDP-43) (32–36), and abnormal translocation with phosphorylation (htt, TDP-43) (2, 9, 17).

Transactivation-responsive DNA-binding protein 43 is normally localized in the nucleus and pathologically translocated to the cytoplasm (9), whereas htt is translocated in the opposite direction: going from the cytoplasm to the nucleus (17). Similar to TDP-43 inclusions in FTLD and ALS, htt inclusions occur in both the cytoplasm and the nucleus. Both proteins are involved in transcriptional regulation. Transactivation-responsive DNA-binding protein 43 was initially reported to repress expression of the human immunodeficiency virus 1 gene (3) and to regulate splicing of the cystic fibrosis transmembrane conductance regulator (4). Huntingtin may be involved in transcriptional regulation of brain-derived neurotrophic factor expression and, more speculatively, with nucleocytoplasm shuttle of transcription regulators. In addition, htt has been implicated in vesicle trafficking, facilitation of axonal transport, and synaptic transmission (for a review, see Imarisio et al (37)). A possible connection of TDP-43 to cytoskeletal stability and axonal transport has been pointed out recently in a study that showed that TDP-43 regulates neurofilament H RNA stability (38).

A major question that remains to be answered is whether both proteins colocalize in the same inclusions because they are involved in common functional pathways or whether the colocalization is incidental to their accumulation after pathological transformation.

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REFERENCES