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Induction of expression and co-localization of heat shock polypeptides with the polyalanine expansion mutant of poly(A)-binding protein N1 after chemical stress

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ABSTRACT

Formation of nuclear inclusions consisting of aggregates of a polyalanine expansion mutant of nuclear poly(A)-binding protein (PABPN1) is the hallmark of oculopharyngeal muscular dystrophy (OPMD). OPMD is a late onset autosomal dominant disease. Patients with this disorder exhibit progressive swallowing difficulty and drooping of their eye lids, which starts around the age of 50. Previously we have shown that treatment of cells expressing the mutant PABPN1 with a number of chemicals such as ibuprofen, indomethacin, $ZnSO_4$, and 8-hydroxy-quinoline induces HSP70 expression and reduces PABPN1 aggregation. In these studies we have shown that expression of additional HSPs including HSP27, HSP40, and HSP105 were induced in mutant PABPN1 expressing cells following exposure to the chemicals mentioned above. Furthermore, all three additional HSPs were translocated to the nucleus and probably helped to properly fold the mutant PABPN1 by co-localizing with this protein.

Oculopharyngeal muscular dystrophy (OPMD) is a late onset, autosomal dominant genetic disease [1]. The largest group of patients with OPMD are present among the French-Canadian population of Quebec, where approximately one in a thousand people is a carrier of OPMD [2]. The clinical symptoms of OPMD usually appear in the fifth decade of life, and patients show progressive swallowing difficulty, eyelid drooping, and limb weakness [1]. Accumulation of nuclear inclusions in OPMD muscle fibres are the hallmark of OPMD [3,4]. Expansion of a polyalanine tract in the nuclear poly(A)-binding protein (PABPN1) is believed to be the underlying genetic mechanism of OPMD [5]. The normal PABPN1 has a repeat of six GCG codon, which encodes the first six alanines of a ten amino acids long alanine tract at the N-terminal end of PABPN1. The other four alanines of the polyalanine tract are coded by the GCA codon. Expansion of the GCG codon to 8–13 repeats leads to an expansion of the polyalanine tract causing aggregation of PABPN1 [6]. Expression of polyalanine expanded PABPN1 in cell culture models showed PABPN1 aggregation, and formation of nuclear inclusions similar to those found in the muscle of OPMD patients [7]. The details of the pathogenic process caused by aggregation of PABPN1 are still not clear. The polyalanine expansion is believed to induce misfolding of proteins in a way similar to that of the polyglutamine expansion-containing proteins [8–10]. It is believed that for polyalanine and polyglutamine expansions, the protein aggregates produce a wide range of toxic effects in cells, and induce apoptosis [1,11]. In addition, PABPN1 aggregate may disrupt cellular functions by sequestering and trapping poly(A) containing RNAs and various transcription factors [6,12,13].

We have earlier shown that induction of the molecular chaperone HSP70 expression in mutant PABPN1 transfected cells by heat shock treatment, and exposure to various pharmacological agents can reduce the aggregate formation [14]. Therefore, these agents could have potential therapeutic values to OPMD patients. However, our earlier studies were limited to the examination of HSP70 and HSC70 expression, and their nuclear translocation.

Abbreviations: PABP(N1), nuclear poly(A)-binding protein; OPMD, oculopharyngeal muscular dystrophy; GFP, green fluorescent protein; HSP, heat shock polypeptide.

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Therefore, to further examine whether induction of the expression and nuclear translocation of additional molecular chaperones such as HSP27, HSP40, and HSP105 were also involved in dissociating the polyalanine expanded mutant PABPN1 aggregates, we have investigated the effect of various drugs on the mutant PABPN1 expressing cells. We report here that in addition to HSP70; $ZnSO_4$, hydroxy-quinoline, ibuprofen, and indomethacin can induce expression of HSP40, HSP27, and HSP105, and localize these HSPs in the nuclei of mutant PABPN1 transfected cells.

Experimental procedures

Culture and transfection of cells. HeLa cells were grown as a monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 10 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Burlington, ON, Canada) at 37 °C in the presence of 5% CO_2. Approximately 3×10^5 cells on a 35 mm dish were transfected with 2 μg of plasmid DNA using lipofectAMINE for 6 h according to manufacturer's protocol (Invitrogen).

Construction and expression of PABPN1-GFP fusion protein. The cDNA clones containing the entire coding regions of both wild type with six GCG repeats (PABPN1-A10), and the mutant with 13 GCG repeats (PABPN1-A17) of human PABPN1 gene were used to create expression vectors encoding PABPN1-A-10 or -A17-GFP fusion proteins [14]. The GFP reading frame was fused at the C-terminal end of PABPN1 in the pEGFP-N3 vector (Clontech, USA).

Confocal microscopy. HeLa cells grown on a glass cover slip were washed with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 20 min. Confocal microscopy was performed to determine the cellular localization of the GFP fusion protein as previously described [14]. The specimen was mounted in PBS containing 70% glycerol and examined with a Leica laser scanning confocal microscope. Approximately 50 cells in each of three separate experiments were examined, and recorded as TIF files. Images representative of the protein localization in greater than 80% cells have been used for presentation.

Induction of HSPs. Forty-eight hours after transfection, cells were treated with different chemicals dissolved in 70% ethanol. The final concentration of $ZnSO_4$, 8-hydroxy-quinoline, indomethacin and ibuprofen in the culture medium was, 50, 50, 150, and 400 μMs, respectively. Following 6 h of exposure to the chemical, cells were allowed to recover in a fresh medium, in the absence of the chemical for 24 h. In some studies cells were heat stressed at 42 °C for 2 h and recovered at 37 °C for 24 h before being examined.

Measurement of protein levels. Cells were washed three times with PBS and lysed by adding 400 μl of gel-loading buffer (50 mM Tris–HCl, pH 6.8, 2% (w/v) sodium dodecylsulfate (SDS), 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol, and 100 mM dithiothreitol [DTT]). To obtain the nuclear and cytoplasmic fractions, cells were washed once with PBS, harvested by scraping, pelleted by centrifugation and lysed in a low salt buffer (20 mM Hepes, pH 7.5, 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.2% Nonidet P-40). Following centrifugation of the cell lysate for 5 min at 1000*g* the supernatant was used as the cytoplasmic fraction. The pelleted nuclei was resuspended in the gel-loading buffer and used as the nuclear extract. The samples were subjected to SDS–12% polyacrylamide gel electrophoresis (PAGE). The separated polypeptides were electrophoretically transferred from a gel to a nitrocellulose membrane, and individual polypeptides were detected by immuno blotting by using the horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) secondary antibody and the Western Blot Chemiluminescence Reagent Plus (PerkinElmer Life Science, MA, USA).

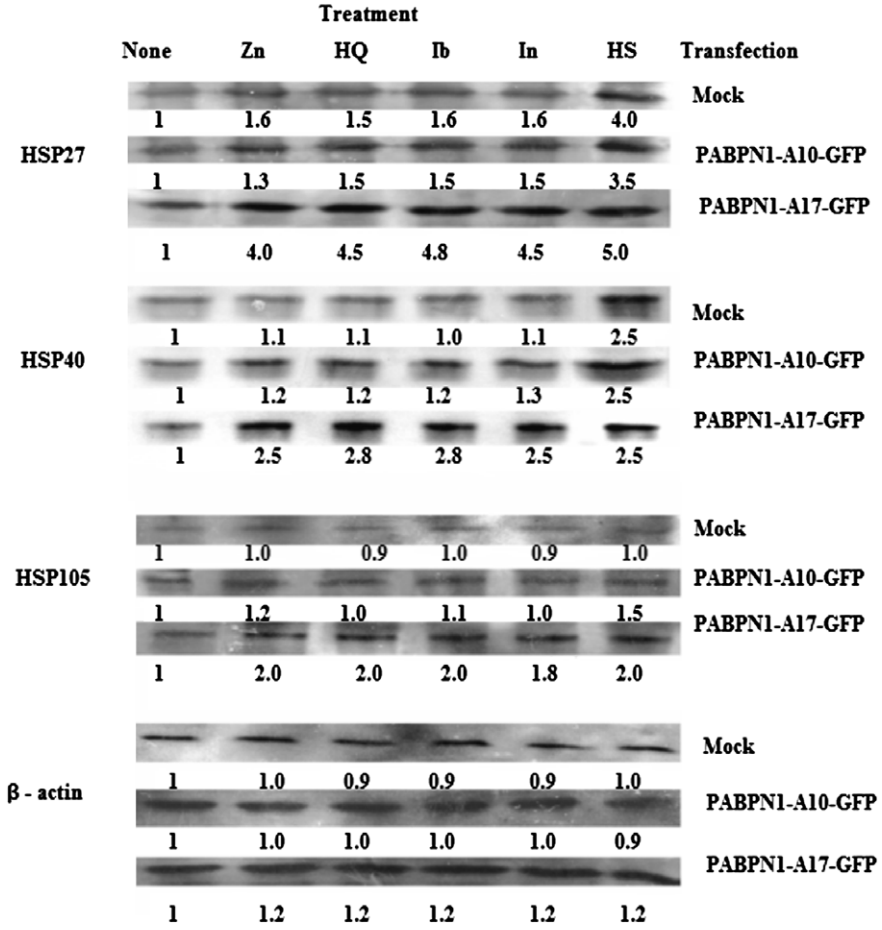

Fig. 1. Changes in the abundance of HSPs. Following 48 h of transfection, cells were treated with different agents or subjected to thermal stress (HS) as described in experimental procedures. Samples containing equal amount of protein were separated by SDS–PAGE and processed for western blotting as described in experimental procedures. Antibodies for HSPs and β-actin were obtained from Santa Cruz Biochemicals. The β-actin level of all samples were measured and used as loading controls. The images were scanned and quantified using scan image software. The normalized values in an arbitrary scale, after correcting for the loading difference are shown at the bottom of each lane. The cellular level of specific polypeptides in untreated transfected and mock transfected cells have been considered as equal to 1. The following abbreviations for different chemicals have been used, $ZnSO_4$ (Zn), 8-hydroxy-quinoline (HQ), ibuprofen (Ib), and indomethacin (In).

Immunostaining of proteins. Cells grown on a cover slip were fixed with methanol at −20 °C for 10 min, and permeabilized with 0.1% Triton X-100 in PBS for 2 min at room temperature as previously described [14]. The fixed cells were treated with 10% normal blocking serum derived from the same species as the primary antibody in PBS for 20 min. The presence of a specific polypeptide was detected with the appropriate antibody. Following 1 h incubation with the primary antibody, the cells were washed in PBS, and incubated with a Texas Red conjugated secondary antibody (1:1000 dilutions, Santa Cruz) for 1 h at 20 °C. The cells were mounted in 70% glycerol in PBS for fluorescent microscopy.

Results

Our previous work [14] has shown that expression of the polyalanine expanded mutant PABPN1 (PABPN1-A17-GFP) in HeLa cells can produce a mild stress response, possibly as a cellular response to refold the mutant PABPN1 aggregates. We have shown in those studies that further enhancement of the stress response by either heat shock treatment or treatment of cells with stress inducing chemicals including, $ZnSO_4$, hydroxy-quinoline, ibuprofen and indomethacin, reduced aggregate formation and cell death [14]. However, in these studies we had only measured the induction of HSP70, and nuclear translocation of HSP70 and the constitutive chaperone HSC70, as a measure for the stress response. Therefore, to analyze whether these agents can invoke a full stress response in PABPN1-A17-GFP expressing cells, the induction of a number of other heat shock proteins including, HSP27, HSP40, and HSP105 was examined by western blotting. The results (Fig. 1) show that compared to the untreated PABPN1-A17-GFP (mutant PABPN1) transfected cells, there were approximately a 2- to 5-fold increase in the cellular abundance of HSP27, HSP40, and HSP105 following treatment with all four chemicals. The induction of HSPs by $ZnSO_4$, hydroxy-quinoline, ibuprofen and indomethacin was similar to those observed by thermal stress. Both untransfected and wild type PABPN1 (PABPN1-A10-GFP) transfected cells showed a less pronounced induction of HSP27, HSP40, and HSP105 expression after exposures to $ZnSO_4$, hydroxy-quinoline, ibuprofen, and indomethacin than what was observed with the mutant PABPN1 expressing cells. Induction of HSPs, and nuclear translocation of both inducible HSPs, and the constitutive chaperone HSC70 are key features of stress response, we examined nuclear translocation of several HSPs in cells after different treatments. In our earlier studies we have reported that both HSP70 and HSC70 translocates from the cytoplasm to the nucleus after treatment of PABPN1-A17-GFP expressing cells with any one of the four agents mentioned above. In the studies reported here we have performed similar analyses for additional HSPs. Results of our analyses (Fig. 2) on the sub cellular distribution of HSP27, HSP40, and HSP105 show that these proteins were predominantly localized into the nuclear fractions of $ZnSO_4$, hydroxy-quinoline, ibuprofen, and indomethacin treated cells. In contrast, prior to the drug treatment, HSP27, HSP40, and HSP105 were predominantly present in the cytoplasmic fraction of non-transfected, wild type, and mutant PABPN1 transfected HeLa cells. However, a low level of HSP27 and HSP105 was detected in the nuclear fraction of untreated cells. Furthermore, the nuclear HSP27 and HSP105 levels were found to be a little higher in PABPN1-A17-GFP transfected cells than what was observed for non-transfected and wild type PABPN1-A10-GFP transfected cells. The nuclear HSP40 was undetectable in the untreated non-transfected and the PABPN1-A10-GFP transfected cells. In contrast, HSP40 was detectable in the nucleus of cells expressing the mutant PABPN1. The sub cellular distribution profiles of all three HSPs studied here in the drug treated mutant PABPN1 expressing cells, were similar to the predominantly nuclear distribution pattern of these HSPs, in PABPN1-A17-GFP expressing cells following a thermal stress.

To analyse how HSP27, HSP40, and HSP105 were distributed in individual transfected cells, we examined cells by confocal

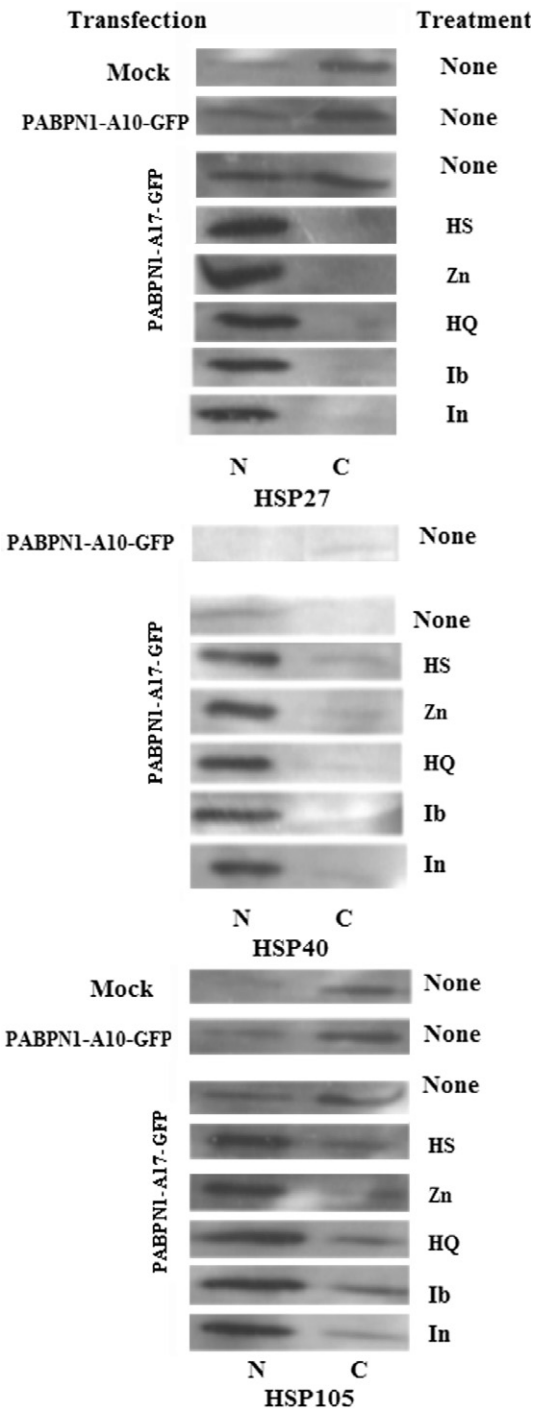

Fig. 2. Sub cellular distribution of HSPs. Transfected and mock transfected cells were treated with different agents, and nuclear and cytoplasmic fractions were obtained as described in experimental procedures. The abundance of different polypeptides was determined by western blotting as described in the legend to Fig. 1.

microscopy following immunostaining. These cells were also examined for co-localization of HSPs with PABPN1-GFP. Here we only presented the subcellualr distribution profiles of HSP27 since the distribution profiles of HSP40 and HSP105 were similar to that of the HSP27 (Supplementary material). The results (Fig. 3) that a low level HSP27 was present in the perinuclear cytoplasm of PABPN1-A10-GFP transfected cells. In the non-transfected cells the perinuclear localization of HSP27 was also detectable; an increase in the abundance of HSP27, and its nuclear

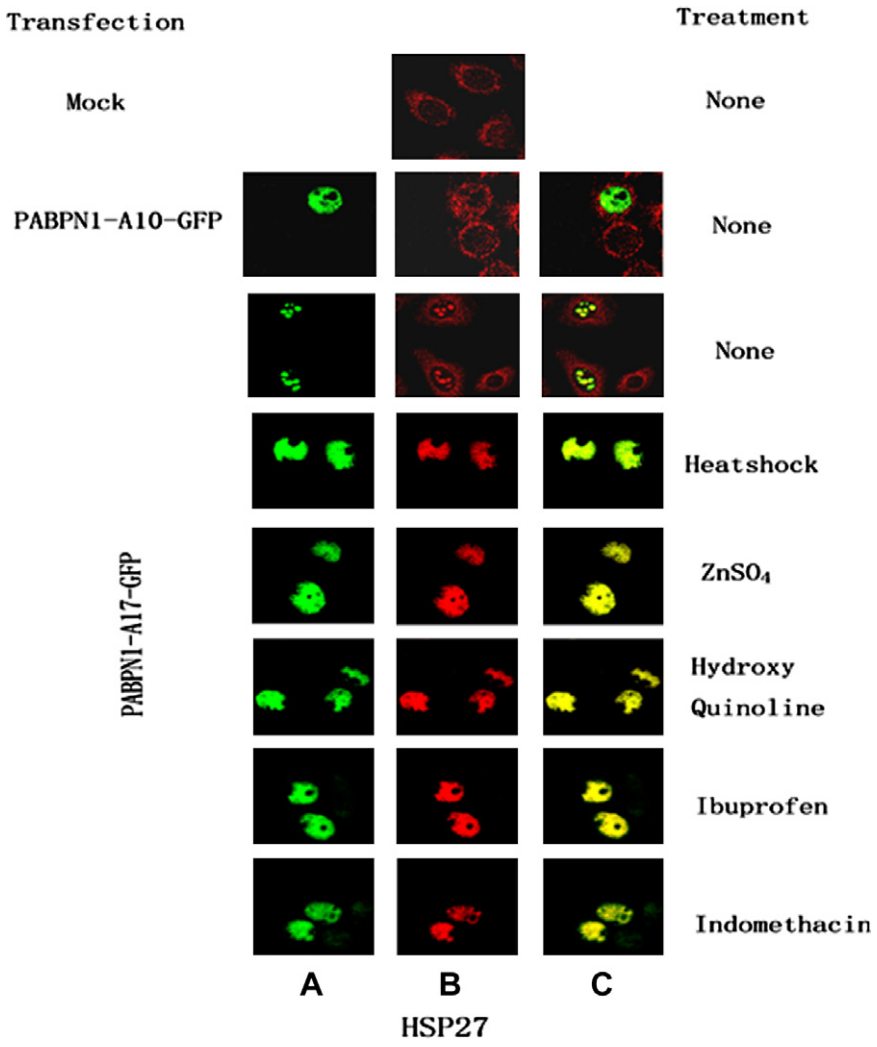

Fig. 3. Cellular localization of HSPs in drug treated transfected cells by immunofluorescence confocal microscopy. Fixed cells were treated with antibody and counter stained with Texas red conjugated secondary antibody as described in experimental procedures. The stained specimens were mounted in the mounting solution and examined under a Leica laser scanning confocal microscope. Approximately 50 transfected cells were examined for each of three separate transfection experiments, and distribution pattern representing 70–80% of cells are shown. (A–C) Show the distribution of green fluorescent tagged PABPN1, the antibody stained HSP27 and red/green merged co-localization of HSP27 with PABPN1-GFP, respectively, in same cells. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

translocation was evident in PABPN1-A17-GFP expressing cells. In addition, HSP27 was found to be co-localized with the mutant PABPN1 aggregates. As previously shown [14], exposure of cells to stress inducing agents including thermal shock changed the nuclear distribution of PABPN1-A17-GFP aggregates to a more diffuse pattern with a concomitant redistribution of HSP27. These studies also revealed that the HSP27 remained colocalized with the diffuse nuclear PABPN1-A17-GFP, suggesting that the HSP27 was probably involved in refolding the PABPN1-A17-GFP aggregates into its native form.

Discussion

HSPs including HSP70, HSP27, HSP40, and HSP105 participate in protein folding and maintaining the native structure of polypeptide [15–17]. The nuclear translocation of several HSPs, and their co-localization with the aggregates of polyalanine expanded mutant PABPN1 suggest that, (i) these aggregates produced a stress response in cells, and (ii) the HSPs interacted with the aggregates in an attempt to refold the mutant protein. However, this response was not sufficiently strong to prevent protein aggregation. Further induction of the HSPs by any of the four chemicals used in our studies showed that increases in the abundance of several HSPs, and their nuclear translocation reduced aggregation of the polyalanine expansion mutant of PABPN1. We found that HSP27, HSP40 and HSP105 remained associated with the PABPN1-A17-GFP following the drug treatment. In a previous report we have shown that both constitutive and inducible HSP70 chaperones also remain associated with the disaggregated PABPN1-A17-GFP. As protein aggregation proceeds through formation of an oligomeric protein [18], we do not know at present whether the HSPs associated with the nuclear PABPN1-A17-GFP, which appeared diffuse, were associated with the oligomeric form of the mutant protein. In our studies we performed the drug treatment for HSP induction, 48 h after transfection, which was sufficient to form nuclear aggregates of ectopic PABPN1-A17-GFP [14]. Therefore, PABPN1 aggregates were present before the HSP induction began following the drug treatment. This suggests that the HSPs were capable of refolding the aggregates that were already present. Further studies are required to determine whether PABPN1 was completely refolded into its native form, or it remained as oligomers.

An important consequence of mutant PABPN1 expression was the trapping of HSP70, HSC70, HSP105, HSP40, and HSP27 in the nuclei with the mutant PABPN1. Thus it is likely that less HSPs

would be available to act as chaperones to fold many normal proteins properly. In a recent study it was shown that expression of polyglutamine aggregates perturbed the global protein folding quality control, which led to the loss of function of many metastable proteins, and consequently caused cell death [19]. This scenario may also be true for polyalanine expanded PABPN1 aggregates due to sequestration of chaperone HSPs. Although, HSPs remained associated with mutant PABPN1 in cells treated with stress inducing chemicals in a diffuse form in the nucleus, significant improvement in cell viability was observed [14], suggesting an improvement of global protein folding due to increased availability of HSPs.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.02.162.

References

[1] B. Brais, Oculopharyngeal muscular dystrophy: a late-onset polyalanine disease, Cytogenet. Genome Res. 100 (2003) 252–260.
[2] G. Brunet, F.M. Tome, F. Samson, J.M. Robert, M. Fardeau, Oculopharyngeal muscular dystrophy, Rev. Neurol. (Paris) 146 (1990) 425–429.
[3] F.M. Tome, V. Askanas, W.K. Engel, R.B. Alvarez, C.S. Lee, Nuclear inclusions in innervated cultured muscle fibers from patients with oculopharyngeal muscular dystrophy, Neurology 39 (1989) 926–932.
[4] F.M. Tome, D. Chateau, A. Helbling-Leclerc, M. Fardeau, Morphological changes in muscle fibers in oculopharyngeal muscular dystrophy, Neuromuscul. Disord. Suppl. 1 (1997) S63–S69.
[5] B. Brais, J.P. Bouchard, Y.Z. Xie, D.L. Rochefort, N. Chretien, F.M. Tome, R.G. Lafreniere, J.M. Rommens, E. Uyama, O. Nohira, S. Blumen, A.D. Korczyn, P. Heutink, J. Mathieu, A. Duranceau, F. Codere, M. Fardeau, G.A. Rouleau, Short GCG expansions in the PABP2 gene cause oculopharyngeal muscular dystrophy, Nat. Genet. 18 (1998) 164–167.
[6] A. Calado, F.M. Tome, B. Brais, G.A. Rouleau, U. Kuhn, E. Wahle, M. Carmo-Fonseca, Nuclear inclusions in oculopharyngeal muscular dystrophy consist of poly(A) binding protein 2 aggregates which sequester poly(A) RNA, Hum. Mol. Genet. 9 (2000) 2321–2328.
[7] A. Abu-Baker, C. Messaed, J. Laganiere, C. Gaspar, B. Brais, G.A. Rouleau, Involvement of the ubiquitin-proteasome pathway and molecular chaperones in oculopharyngeal muscular dystrophy, Hum. Mol. Genet. 12 (2003) 2609–2623.
[8] L.M. Shinchuk, D. Sharma, S.E. Blondelle, N. Reixach, H. Inouye, D.A. Kirschner, Poly-(L-alanine) expansions form core beta-sheets that nucleate amyloid assembly, Proteins 16 (2005) 579–589.
[9] J.P. Tavanez, P. Calado, J. Braga, M. Lafarga, M. Carmo-Fonseca, In vivo aggregation properties of the nuclear poly(A)-binding protein PABPN1, RNA 11 (2005) 752–762.
[10] K. Mitsui, H. Doi, N. Nukina, Proteomics of polyglutamine aggregates, Methods Enzymol. 412 (2006) 63–76.
[11] C. Landles, G.P. Bates, Huntingtin and the molecular pathogenesis of Huntington's disease, EMBO Rep. 5 (2004) 3–10.
[12] Q. Wang, J. Bag, Expression of a polyalanine expansion mutant of poly(A) binding protein in cells in culture inhibits myogenesis, Biochem. Biophys. Res. Commun. 40 (2006) 815–820.
[13] A. Abu-Baker, G.A. Rouleau, Oculopharyngeal muscular dystrophy: recent advances in the understanding of the molecular pathogenic mechanisms and treatment strategies, Biochem. Biophys. Acta 1772 (2007) 173–185.
[14] Q. Wang, D.D. Mosser, J. Bag, Induction of HSP70 expression and recruitment of HSC70 and HSP70 in the nucleus reduce aggregation of a polyalanine expansion mutant of PABPN1 in HeLa cells, Hum. Mol. Genet. 14 (2005) 3673–3684.
[15] C.Y. Fan, S. Lee, D.M. Cyr, Mechanisms for regulation of Hsp70 function by Hsp40, Cell Stress Chaperones 8 (2003) 309–316.
[16] C. Garrido, M. Brunet, C. Didelot, Y. Zermati, E. Schmitt, G. Kroemer, Heat shock proteins 27 and 70: anti-apoptotic proteins with tumorigenic properties, Cell Cycle 5 (2006) 592–601.
[17] T. Hatayama, K. Ysuda, E. Nishiyama, Characterization of high-molecular-mass heat shock proteins and 42 degrees C-specific heat shock proteins of murine cells, Biochem. Biophys. Res. Commun. 204 (1994) 357–365.
[18] D.C. Rubinsztein, J.A. Huntigton, Paradoxical aggregation versus oligomerisation properties of mutant and wild-type huntingtin fragments, Exp. Neurol. 199 (2006) 243–254.
[19] T. Gidalevitz, A. Ben-Zvi, K.H. Ho, H.R. Brignull, R.I. Morimoto, Progressive disruption of cellular protein folding in models of polyglutamine diseases, Science 311 (2006) 1471–1474.