



Intracellular FMRpolyG-Hsp70 complex in fibroblast cells from a patient affected by fragile X tremor ataxia syndrome

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ABSTRACT

Background: Fragile X-associated tremor/ataxia syndrome is a late-onset neurodegenerative disorder that affects about 40% of carriers of CGG-repeat expansions in the premutation range within the fragile X gene (*FMR1*). Main clinical features include intention tremor, cerebellar ataxia, and parkinsonism. Recently, great emphasis on the deposition of soluble aggregates produced by a RAN translation process, as main pathogenic mechanism, has been given. These aggregates contain a small protein with a polyglycine stretch on the aminoterminal end named FMRpolyG and, so far, have been isolated and characterized in drosophila and mouse models, in post mortem brain of fragile X-associated tremor/ataxia syndrome patients, in fibroblasts of fragile primary ovarian insufficiency patients, but never in fibroblasts from a fragile X-associated tremor/ataxia living patients. In adult carriers the syndrome is frequently misdiagnosed due to the lack of specific markers.

Methods: We standardized immunocytochemistry, immunoprecipitation and western blot procedures to study and biochemically characterize the FMRpolyG protein in fibroblasts from human skin biopsy.

Results: We demonstrate for the first time, in fibroblasts from a patient affected by Fragile X-associated tremor/ataxia syndrome, the presence ex vivo of inclusions consisting of FMRpolyG- Hsp70 soluble aggregates.

Conclusion: These observations can pave the way to develop a cellular model for studying ex vivo and in vitro the mechanisms involved in the production of FMRpolyG aggregates, their toxicity, and the role of the FMRpolyG-Hsp70 interaction in the pathogenesis of fragile X-associated tremor/ataxia syndrome.

1. Introduction

Fragile X-associated tremor/ataxia syndrome (FXTAS) is a late-onset neurodegenerative disorder that affects about 40% of males and 16% of females carriers of CGG-repeat expansions in the premutation range (55–200 repeats), within the fragile X gene (*FMR1*). Main clinical features of FXTAS include intention tremor, cerebellar ataxia, and parkinsonism [Hagerman et al., 2001; Hall et al., 2014]. Premutations in the *FMR1* are also responsible of primary ovarian insufficiency (FXPOI) in approximately 20% of carriers females [Man et al., 2017]. While the full mutated allele (>200 repeats) is completely silenced, the premutation is characterized by a significant up regulation of the mRNA leading to a condition of RNA toxicity [Usdin et al., 2014]. Recently, great emphasis on the pathogenic role of protein toxic inclusions, produced by a repeat

ATG non dependent translation process (RAN) on the *FMR1* 5' expanded CGG region, has been given [Kearse and Todd, 2014; Sellier et al., 2017]. These ubiquitin positive inclusions, composed of a small protein of about 15 KD with an aminoterminal polyglycin stretch (FMRpolyG) and chaperone proteins such as Hsp70, have been isolated and characterized in drosophila and mouse models, in living FXPOI patients, in post mortem FXTAS patients biopsies [Greco et al., 2002], but never in FXTAS living patients [Hagerman and Hagerman, 2015]. The lack of specific markers in adult carriers of premutations makes the early diagnosis of FXTAS challenging [Rajan-Babu and Chong, 2016], and the complexity of the procedure aimed at producing human cellular models, makes the study of the role of these inclusions in the pathogenesis of FXTAS difficult as well [Sheridan et al., 2011].

Using Immunocytochemistry (ICC), Immunoprecipitation (IP) and

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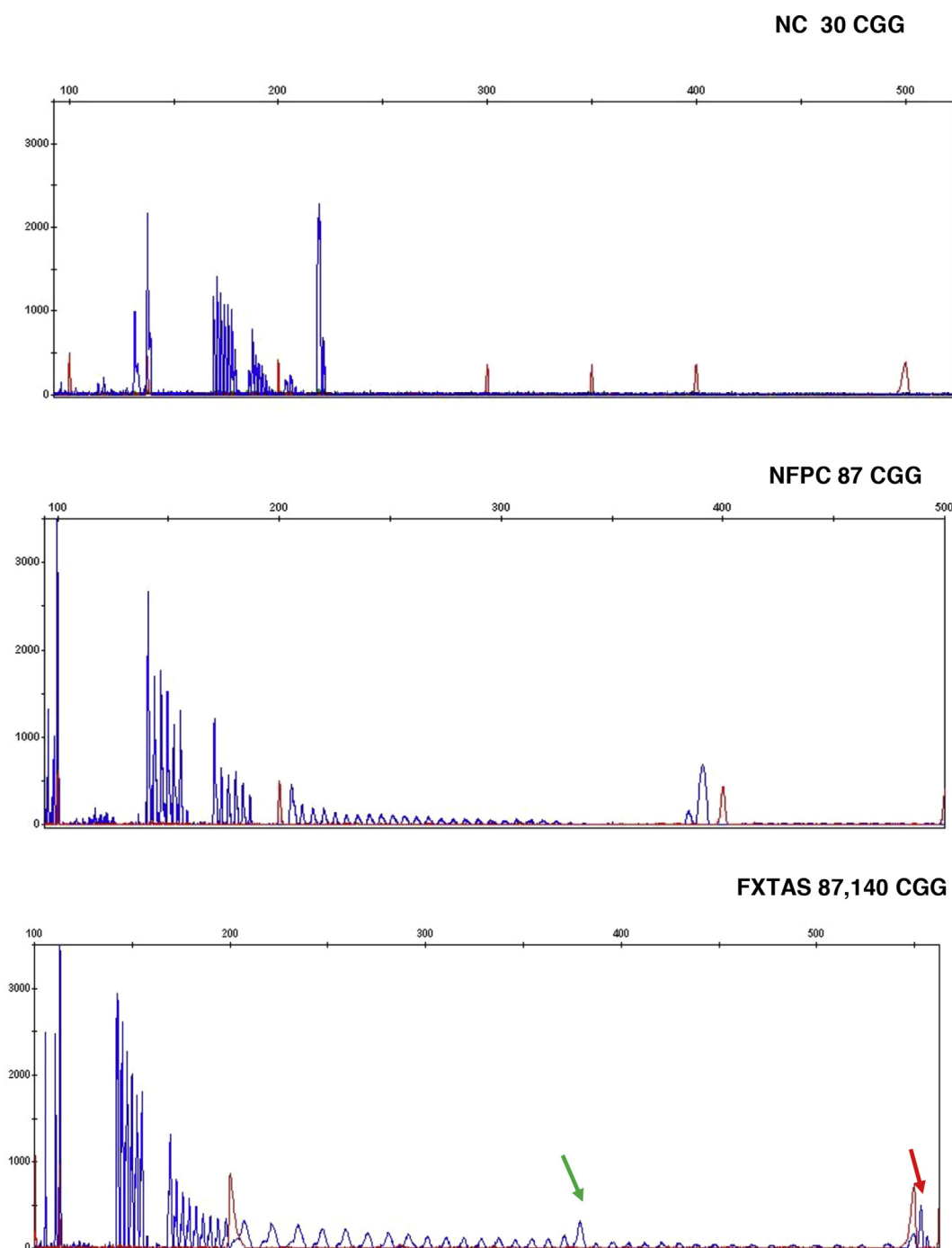


Fig. 1. Genescan analysis of the CGG repeats within FMR1 gene from: Normal Male control (NC), Non FXTAS premutated carrier (NFPC), and FXTAS patient Allele sizes are shown in the upper right corner. The NFPC was carrier of an 87 CGG allele, the FXTAS patient was a mosaic for an 87 CGG allele and an 140 CGG allele. The upper panel shows the normal control (NC) with a 30 CGG allele.

Western Blot (WB) procedures, we have been able to demonstrate the presence of inclusions containing FMRpolyG-Hsp70 complexes in fibroblasts from skin biopsy of a clinical and molecular characterized FXTAS living patient, and discuss some possible practical implications.

2. Materials and Methods

2.1. Patient and controls

All experiments involving human subjects were conducted in accordance with the Declaration of Helsinki <http://www.wma.net> and all the

procedures were carried out with the adequate understanding and written consent of the subjects.

Patient was a 50 years old male, with a history of tremor and slowness since age 43. He also complained of occasional syncopal episodes. His past and family histories were unremarkable. Neurological examination revealed broad-based gait, facial hypomimia, mild asymmetric (right greater than left) resting tremor in the distal upper and lower extremities, in association with postural and action tremor in the upper limbs. Tone, strength, and sensation were normal. Slight bradykinesia and increased deep tendon reflexes were also present. General physical examination was normal, without sexual or urinary disturbances, except for

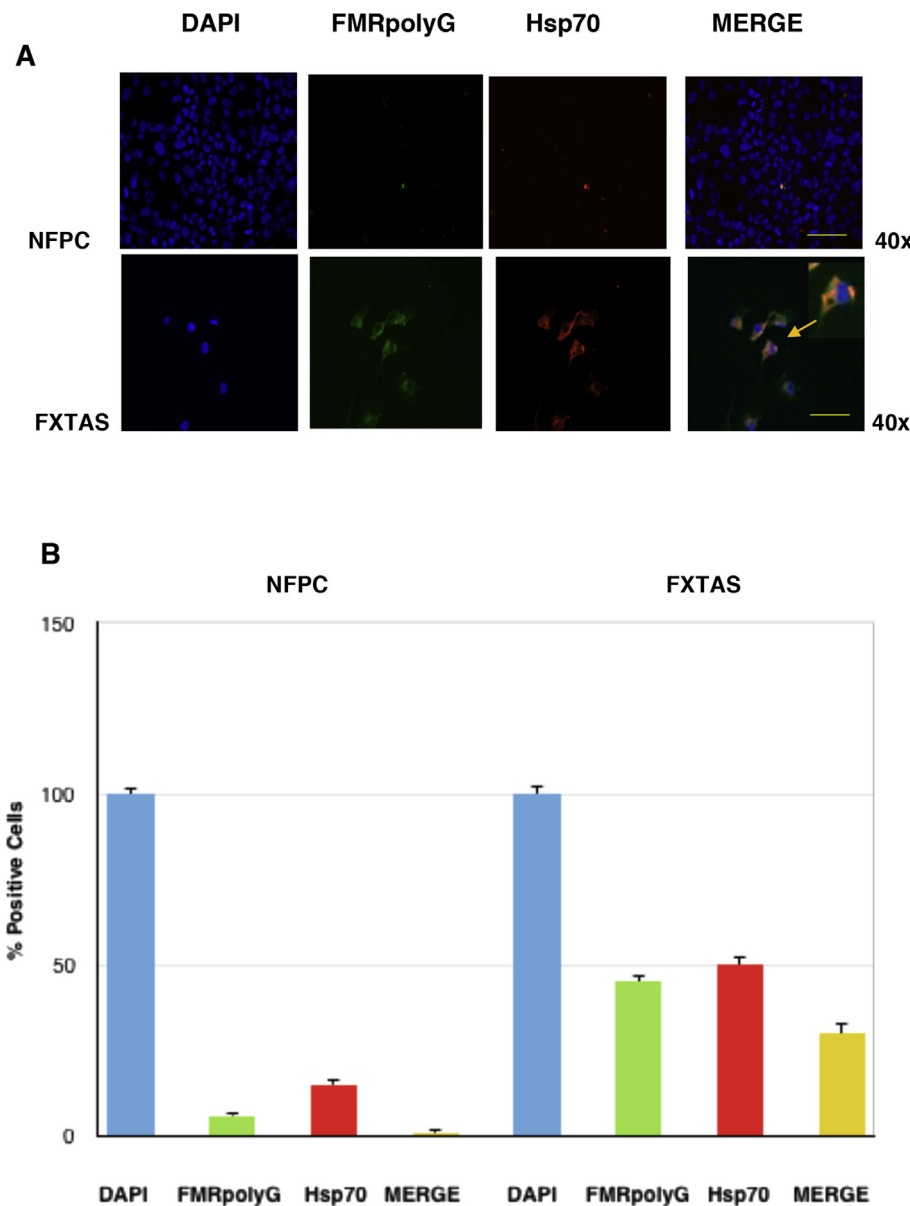


Fig. 2. In fibroblasts of FXTAS patient FMRpolyG colocalizes with Hsp70 **A:** Fibroblasts from FXTAS patient and NFPC were processed for indirect immunofluorescence staining using primary antibodies against FMRpolyG (green) and Hsp70 (red). **B:** Averaged data from three experiments. About 45+/-3% of the FXTAS cells were positive for FMRpolyG and only 8+/-2% in NFPC ($p < 0,001$). Strikingly, 50+/-1% of the FMRpolyG positive cells showed a clear colocalization with Hsp70 (see inset Merge). No colocalization was evident for NFPC.

orthostatic hypotension with a decrease in systolic and diastolic blood pressure of 34 and 14 mmHg respectively, after changing position from supine to standing upright. A generalized cerebral atrophy with severe involvement of the cerebellum and of the middle cerebellar peduncles were present in 3T MRI scan. The non FXTAS premutated control (NFPC) was a premutated healthy 40-year-old male, without clinical sign of FXTAS or Fragile X Syndrome (FXS). 3T MRI scan and neurological examination were normal. His younger brother was affected by FXS, associated to a 250 repeats full mutated allele. The normal control (NC) was a healthy male, 50 years old without pathological expansions on *FMR1* gene.

2.2. Gene scan analysis

Depending on the size of the CGG repeat region, different amplicons were produced using a specific FXS Triple Primed PCR Kit (Abbott Molecular code: 6L4303). Hi-Di formamide (Applied Biosystems), and 2µl of ROX 350 size standard (Celera Corporation) were combined with 2µl of PCR product. Samples were denatured at 95 °C for 2 min before loading onto an ABI 310 with POP-6 polymer on a 36-cm or 50-cm array. Data

were analyzed using the Gene Mapper v.3.7 software.

2.3. Immunocytochemistry

Fibroblasts from NC, NFPC and patient, collected by skin biopsies, were grown in RPMI plus 10% FBS. Cells were trypsinized at confluence, plated in chamber slide at 2×10^6 density (Nunc Rochester NY) and incubated 48 h in RPMI 10% FBS. After paraformaldehyde treatment, permeabilization with 0.2% Triton X-100 followed by blocking in 0.2% gelatin at 4 °C, cells were incubated for 3h at room temperature with an anti FMRpolyG monoclonal antibody (clone 9FM 1-B7 Millipore USA) and a goat anti human Hsp70 1:200 (Santa Cruz). An anti human ubiquitin rabbit monoclonal antibody (EPR8830, Abcam) was used for Ub ICC. Anti-mouse FITC conjugated, anti-goat TRITC conjugated and anti rabbit TRITC conjugated specific secondary antibodies were used for the detection. Hundred cells for microscopic fields, in a total of hundred different fields, were analyzed. Cells positive for FMRpolyG and for Hsp70 were counted and evaluated as percentage of the total cells in the fields (DAPI staining). All the experiments were in triplicate.

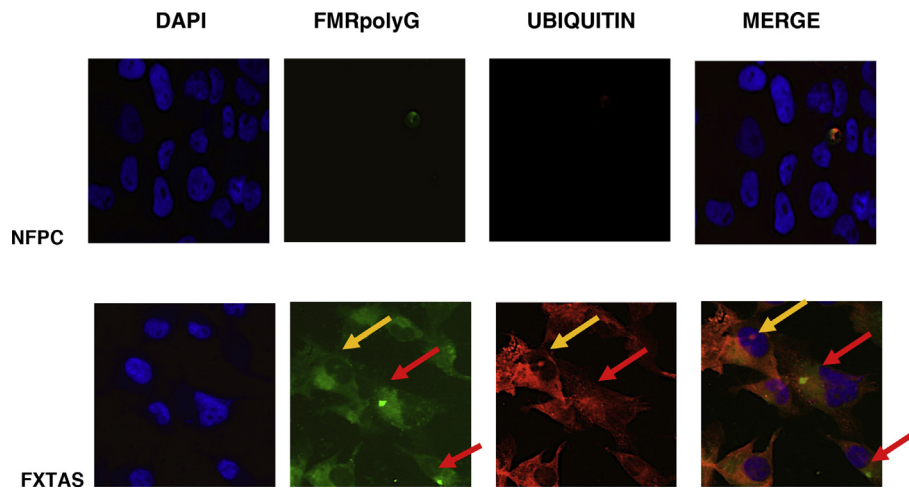


Fig. 3. In fibroblasts of FXTAS patient FMRpolyG nuclear inclusions are ubiquitinated. Fibroblasts from NFPC and FXTAS patient were processed for indirect immunofluorescence staining using primary antibodies against FMRpolyG (green) and against Ubiquitin (red). Yellow arrows show intranuclear inclusion. Red arrows show cytoplasmic inclusions. Only nuclear inclusions are positive for ubiquitin staining (Merge).

2.4. Immunoprecipitation studies

After precleaning with 60 μ l of a Staphylococcus A membrane (Sigma S 2014) 100 μ g of cellular extracts (FXTAS patient, and NFPC), were incubated overnight at 4 $^{\circ}$ C with a rabbit anti human Hsp70 antibody (Hsp70 bound FMRpolyG) or pre immune antisera (unbound FMRpolyG). The immunocomplexes were precipitated by Protein A sepharose beads (Sigma P3391), collected by centrifugation, washed twice with cold PBS, separated by boiling, and analyzed by western blot.

2.5. Western blot analysis

After running on a 12% SDS Page, samples from IP were transferred to a nitrocellulose membrane and analyzed using a primary mouse anti human FMRpolyG and a secondary goat antimouse HRP conjugated (Cell Signaling Technology). Samples immunoprecipitated with rabbit pre immune sera (Serotec) or crude extracts were used as negative controls. In a second set of experiments, a rabbit anti Hsp70 antibody (Santa Cruz Biotechnology) and a secondary anti rabbit HRP conjugated (Purity Anti Rabbit HRP PU 4300020 Vilber Lourmat), were used to assess the amount of the steady state levels of Hsp70 in crude lysates from NFPC and FXTAS patient. Alfa actin (Promega) was used as loading control. The relative protein levels were quantified using ImageJ software.

2.6. Statistical analysis

A Kruskal-Wallis test was used to evaluate significant differences in the number of cell positive for FMRpolyG and for FMRpolyG-Hsp70 complex between NFPC and FXTAS patient.

3. Results

3.1. Genetic analysis

The genetic analysis of the *FMR1* CGG repeats in the FXTAS patient revealed mosaicism of an 87 and 140 CGG expansions in the premutation range for FXS. The NFPC was carrier of a premutated allele of 87 CGG repeats. As expected, no expansions on NC were detected (Fig. 1).

3.2. In the FXTAS patient, mosaicism for 87 and 140 repeats is associated to FMRpolyG inclusions

To assess ex vivo the presence of FMRpolyG protein we performed immunocytochemistry experiments on NC, FXTAS and NFPC fibroblasts.

Fig. 2 shows that the FMRpolyG accumulates as inclusions in fibroblasts of the FXTAS patient, both in the cytoplasm and nucleus (yellow arrow). As expected in presence of an abnormal deposition of protein, there is a clear colocalization of FMRpolyG with the heat shock protein 70 (Hsp70), a well known cellular chaperone involved in the protection against the effects triggered by aggregates (see Inset Merge). No signal for FMRpolyG was detected in NC fibroblast (data not shown).

3.3. In FXTAS fibroblasts, FMRpolyG inclusion are ubiquitinated

Post-mortem neuropathological examination revealed the presence of ubiquitin FMRpolyG positive protein as component of the intranuclear inclusions in neurons and astrocytes, suggesting that such inclusions likely reflect a failure in proteasomal degradation of nuclear proteins [Iwahashi et al., 2006]. To assess if in our model a failure in the proteasomal pathway could be involved in the deposition of the soluble aggregates, we studied the ubiquitinylation status of the FMRpolyG inclusion by ICC. Differently from the specific intranuclear localization described for the inclusions found in post mortem brain tissue, in ex vivo FXTAS fibroblasts, the FMRpolyG protein accumulates both in the cytoplasm in a not ubiquitinated form, and in the nucleus in a slightly ubiquitinated form (Fig. 3).

3.4. In FXTAS fibroblasts, Hsp70 is a component of the FMRpolyG inclusions

To demonstrate that the Hsp70 does not merely colocalize with FMRpolyG, but that it is a component of the same intracellular complex involved in a defensive reaction against the aggregates, we performed coimmunoprecipitation experiments on cellular extracts from FXTAS and NFPC fibroblasts.

Fig. 4 shows that the anti Hsp70 antibody is able to coimmunoprecipitate the FMRpolyG protein, confirming that there is a specific physical interaction with FMRpolyG as described in the first step of proteasomal mediated degradation pathway [Shiber and Ravid, 2014].

Interestingly, the amount of free FMRpolyG in the crude FXTAS cell extract (Fig. 4A, FXTAS -IP) is less than the amount of coimmunoprecipitated protein after incubation with anti Hsp70 (Fig. 4A FXTAS + IP), suggesting that most of the FMRpolyG protein is rapidly sequestered by the defense reaction triggered by Hsp70.

According to this observation, the Hsp70 levels evaluated by WB on the same amount of total proteins are increased in the crude lysate of FXTAS fibroblasts in comparison to the steady state levels in NFPC lysate (Fig. 4B). In the negative control, the presence of a faint signal in WB for

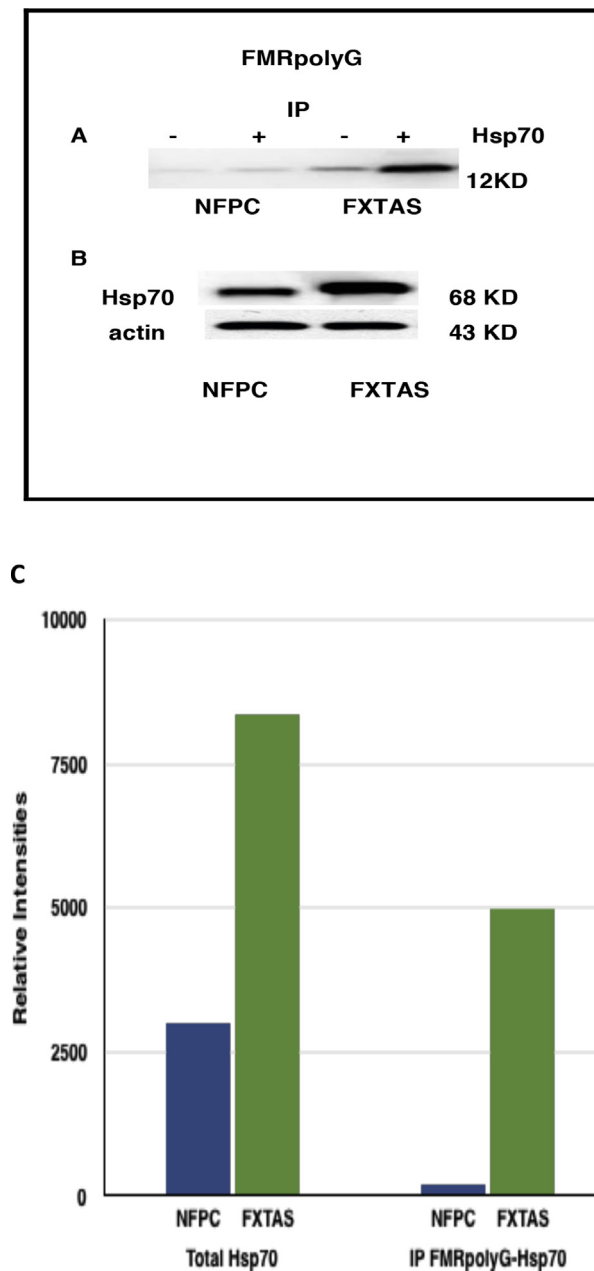


Fig. 4. Recruitment of FMRpolyG by Hsp70 cellular chaperone A: Cellular extracts from FXTAS patient and NFPC fibroblasts were incubated with anti Hsp70. After separation on 12% polyacrilamide gel, the FMRpolyG unbound (L IP) and the bound by Hsp70 (+IP) were blotted and incubated with an anti FMRpolyG Ab. B: Total levels of Hsp70 evaluated by WB on the same amount of total proteins in crude lysate of NFPC and FXTAS fibroblasts C: Signals for total Hsp70 in crude lysate and for FMRpolyGLHsp70 immunocomplexes were analyzed by an image analyzer and the relative band intensities plotted. Strikingly, the recruitment of FMRpolyG by Hsp70 and the previously observed increased levels of total Hsp70 in FXTAS by ICC, were confirmed. LIP: pre immune antisera (unbound FMRpolyG) +IP: HsP70 bound FMRpolyG.

FMRpolyG (Fig. 4A NFPC +/- IP), is due to a slight cross reactivity of the Anti FMRpolyG (clone 9FM 1-B7 Millipore USA) we used and already described [Buijsen et al., 2014].

4. Discussion

In the past years, research on *FMR1* gene has been focused on the individuals with the full mutation and fragile X syndrome (FXS) while

carriers of premutation were thought to be unaffected. Recent clinical studies in premutation carriers have expanded the health problems that may be seen, identifying a broad range of neurological, neurocognitive, endocrine and psychiatric problems such as FXTAS and FXPOI [Polussa et al., 2014].

Features of FXTAS overlap many other neurodegenerative disorders, including Parkinson, Alzheimer diseases and Frontotemporal Dementia (e.g., progressive cognitive impairment, altered mood and behavior) [Fraint et al., 2014]. These observations underscore the need to develop a new and reliable diagnostic tool, to confirm the diagnosis of FXTAS. Kearse and colleagues first demonstrated, on postmortem biopsies and murine models, as a translation of CGG repeats, without the canonical ATG starting codon (RAN), occurs in two out of the three frames, giving rise to short proteins containing either a polyalanine or a polyglycine stretch. Interestingly, only the polyglycine protein resulted in the formation of heterogeneous inclusions, which were toxic both in neuronal transfected cells and in drosophila [Kearse and Todd, 2014].

Based on the above described results of the study done by Kearse et al. we proposed to assess the presence of a such protein in culture of fibroblasts obtained from skin biopsies of a living FXTAS patients, in order to establish a cellular model for studying ex vivo the progression of the disease. Starting from the observation that the FMR1 protein is expressed in a wide range of cell type (<https://www.genecards.org>), we demonstrate, the presence of slightly FMRpolyG ubiquitinated inclusions associated to HsP70 in fibroblasts from a living patient carrier of a pre-mutated *FMR1* allele associated to clinical signs of FXTAS, but not in a non FXTAS premutated carrier of CGG expansion. To our knowledge this is the first observation in a living patients.

In contrast to the single nuclear localization found in post mortem FXTAS tissue, in our model the inclusions are both cytoplasmic and nuclear. According to the studies performed by the group of Lianwu [Fu et al., 2005], this difference, in our living cells, could be due to an ongoing progressive spatial rearrangements and reposition of initial small mobile soluble cytoplasmic inclusions. In fact, the authors describe as, owing to a progressive coalescence and fusion process, in living cells, soluble inclusion are first moved from the cytoplasm and deposited as insoluble aggregates into the nucleus interfering with the normal nuclear and cytoplasmic processes (transcription, hnRNA maturation, protein synthesis), finally leading to cell death. If this is the case, the presence of inclusion only in the nucleus of post mortem tissues represents the final status of a dynamic toxic process, that starts in the cytoplasm and whose progression ex vivo, our cell mode will allow to be studied.

Iwahashi and colleagues, described an increase in Hsp70 levels as marker of cellular defense against the heterogeneous inclusions found in neurons and astrocytes from FXTAS post mortem brain. In another paper, the same group demonstrated as ubiquitinated proteins represent only a minor component of the inclusion [Arocena et al., 2005]. We studied such reactions in the fibroblasts of our patient. Our data demonstrating an increase in the amount of total Hsp70, an evident molecular interaction between the FMRpolyG protein and Hsp70, and a low level of ubiquitinylation, agree with the observations of Iwahashi and Arocena, and confirm also in a living patient, the presence of a protective cellular response against the inclusions without a breakdown in the proteasomal system. Strikingly, in our model, the presence of inclusions both in the cytoplasm and in the nucleus that are slightly ubiquitinated only into the nucleus, seems to confirm the progressive toxicity hypothesis of Lianwu, indicating that the incorporation of ubiquitin might be the last event in the progression that lead to the cellular toxicity and then to the pathogenesis of the FXTAS.

If this is the case, the well documented increase in the production of the Hsp70 protein, its interaction with FMRpolyG, associated to the presence of ubiquitinated inclusion only into the nucleus we observed ex vivo in the FXTAS patient, could have a potential diagnostic and prognostic value.

This is a very important issue because experiments performed on human induced pluripotent stem cell models of FXS/FXTAS, aimed at

studying the step by step development of the disease and to identify early markers of progression, have been not conclusive [Sheridan et al., 2011]. Experiments on more patients are in progress to address this point.

5. Conclusion

Methods based on post mortem biopsies cannot be exploited to study the progression of the FXTAS. The recently described human induced pluripotent stem cell model is time expensive and not completely affordable, due to the observed genetic differences between the original CGG expansion in the input cells and the derived neuronal cell lines. Our data demonstrate for the first time that it is possible to assess the presence of potential toxic FMRpolyG-Hsp70 inclusion in skin biopsies from an FXTAS living patient by using a combined approach based on well consolidated cell culture, IP, ICC and WB procedures. This approach can be used to establish a simple and reproducible human cellular model to study, ex vivo and in vitro, the mechanisms involved in the deposition of the soluble aggregates described as potentially cytotoxic in course of FXTAS, and suggests that in a close future once validated on more cases, this method could be exploited to confirm the diagnosis of the disease in patients showing neurological signs of FXTAS associated to *FMR1* CGG premutated alleles.

Declarations

Author contribution statement

Giuseppe Bonapace: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Rosa Gullace, Daniela Concolino, Grazia Annesi, Gennarina Arabia, Aldo Quattrone: Analyzed and interpreted the data.

Rosa Gullace, Grazia Iannello, Radha Procopio, Monica Gagliardi: Performed the experiments.

Gaetano Barbagallo, Angela Lupo, Lucia Ilaria Manfredini: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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