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# Dual role of p53 amyloid formation in cancer; loss of function and gain of toxicity

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ABSTRACT

The tumor suppressor p53 plays an important role in genome integrity. It is frequently mutated in all types of human cancers, making p53 a key factor in cancer progression. Two phenotypic consequences of these alterations are dominant; a loss of function and a gain of function of p53, which, in several cases, accumulates in intracellular aggregates. Although the nature of such aggregates is still unclear, recent evidence indicates that p53 can undergo conformational transitions leading to amyloid formation. Amyloid diseases, such as, Alzheimer's disease, are characterized by the accumulation of insoluble aggregates displaying the fibrillar conformation. We decided to investigate the propensity of wild type p53 to aggregate and its consequent assembly into different amyloid species, such as oligomers and fibrils; and to determine if these changes in conformation lead to a loss of function of p53. Furthermore, we analyzed cases of Basal Cell Carcinoma (BCC), for the presence of p53 amyloids. Here, we show that p53 forms amyloid oligomers and fibrils, which coincide with p53 inability of binding to DNA consensus sequences. Both p53 amyloid oligomers and fibrils were detected in BCC cancer samples. Additionally, we demonstrate that p53 oligomers are the most cytotoxic to human cell cultures.

Our study reveals p53 amyloid formation and demonstrates its dual role in the pathogenesis of cancer by producing a loss of protein function and a gain of toxic function, extensively described in several amyloidogenic diseases. Our results suggest that under certain circumstances, cancer could be considered a protein-conformation disease.



## 1. Introduction

Mutations in the p53 gene are detected in ~50% of human cancers [1]. In addition to mutant p53 being inactive, several types of human cancers harbor a genetically wild-type, but a transcriptionally inactive form of p53 [2]. In these cases, wild-type p53 has been reported to accumulate in the cytoplasm and/or the nucleus [3]. Moreover, in these cancers, the inactive wild type p53 forms large protein aggregates [4], which may occur due to a conformational change of p53 [5].

A vast range of human diseases arise from the failure of a specific protein to adopt or remain in its native functional conformational state and change into a β-sheet conformation. These pathologic conditions collectively are referred to as protein conformational or amyloid diseases [6]. Recent research suggests that prefibrillar aggregates, soluble oligomers, rather than fibrils per se are the most potent mediators of cytotoxicity [7]. Amyloid fibril deposition has been described in patients with malignant diseases. It has been more frequently seen in hematological neoplasms and has also been noticed in patients with solid tumors. Previous studies demonstrated the presences of amyloid deposition in different types of cancer [8–10] . Amyliod deposition also occurs in numerous benign and malignant epidermal lesions such as basal cell carcinoma (BCC) [11]. The mechanism of amyloid formation in association with solid tumors is unknown and thus further investigation is necessary.

As mentioned above in some cancers, the inactive form of p53 shapes large protein aggregates [4], which can occur secondarily to a conformational change of p53 [5] and may possibly display amyloid-like structures. *In vitro* studies shows how p53 aggregates in amyloid assemblies and induces cytotoxicity in neuroblastoma cells [12], demonstrating that p53 amyloid can behave like any other amyloid disease [13]. So far, only a couple of studies have demonstrated cytoplasmatic inclusions of p53 in Tg mice expressing mutant p53 and in human colon carcinoma [14]. Another study shows the presence of p53 aggregates in human cases of breast cancer [15].

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The present study describes the dual role of p53 amyloid formation in cancer, where there is a loss of protein function and a gain of toxic function of p53. In order to display this "dual role" concept of p53, we analyzed human cases of BCC, which is the most commonly diagnosed malignant skin tumor [16]. Our data show that full length p53 can adopt diverse amyloid conformations such as oligomers and fibrils, and that these amyloid species lose the ability to bind to DNA. Also following a pro-inflammatory stimulus, p53 aggregates and forms amyloid oligomers and fibrils in BCC cell culture. Interestingly, these oligomers and fibrils are also present in human cases of BCC and p53 oligomers are associated with cell toxicity. Overall, our study reveals that p53 aggregation could contribute to disease pathology by not only a gain-of-toxic-function mechanism, but also a partial loss of function mechanism.

## 2. Materials and methods

### 2.1. Dot blot

Human recombinant p53 was purchased from Sigma–Aldrich (Cat #p6374). Fibrils and soluble oligomers were prepared as previously described [17]. Two microliters of each sample was applied to a nitrocellulose membrane, blocked with 5% non-fat milk overnight at 4 °C and then incubated again for 1 h at room temperature with the anti-oligomer antibody A-11 (1:1000) or the anti-fibril antibody OC (1:5000). Then the membranes were incubated with HRP conjugated anti-rabbit IgG (Promega) (1:10,000). Blots were developed with ECL chemiluminescence kit from Amersham-Pharmacia.

### 2.2. Atomic force microscopy (AFM)

The morphology of oligomers and fibrils preparations were assessed by AFM by a non-contact tapping method (ScanAsyst-air) using a Multimode 8 AFM machine (Veeco, CA).

### 2.3. Thioflavin T assay

Twenty μl of samples (3.3 μM) were incubated 15 min at room temperature in 50 mM glycine, pH 9.2 and 2 μM ThT. Fluorescence was measured at excitation wavelength of 435 nm with emission at 485 nm.

### 2.4. DNA binding assay

Fibrils and oligomers were prepared as described above. DNA binding was measured by the Panomics ELISA transbinding kit (Panomisc #EK1050).

### 2.5. Detection of p53 amyloid oligomers and fibrils in Basal Cell Carcinoma cell culture

BCC cells were treated with 0.0005% formaldehyde for 72 h. Then, the cells on the coverslip were fixed and permeabilized. The samples were blocked for 1 h in 5% goat serum and incubated overnight at 4 °C with A-11 (1:1000) or OC (1:5000). Sections were then washed and incubated with an Alexa 568-conjugated goat anti-rabbit antibody (1:700; Invitrogen) for 1 h at room temperature. For co-localization studies, the sections stained with conformational antibodies were then incubated overnight at 4 °C with anti-p53 (1:2000), then washed and incubated with an Alexa 488-conjugated goat anti-mouse antibody (1:700). For nuclear staining, DAPI was used (1:4000, Invitrogen). Sections were then washed for 30 min and cover slipped. Single images of immunostained cells were acquired on an LSM510 Zeiss laser scanning confocal microscope.

### 2.6. Human histology and immunohistochemistry

Biopsy tissue was obtained from the skin of 6 patients with BCC. H&E staining was performed to examine general skin morphology and basal cell carcinoma pathology. Congo red and Thioflavin-S was used to analyze the deposition of amyloid in patients with BCC. The bright field and polarized images were acquired using a Nikon Multizoom AZ100 microscope.

### 2.7. Immunohistochemistry and Immunofluorescence

Immunohistochemistry was performed on paraffin-embedded sections. In brief, primary antibodies were detected with biotinylated goat anti-mouse IgG (1:2000, Jackson ImmunoResearch) or biotinylated goat anti rabbit IgG (1:1800) and visualized with Avidin–Biotin Complex kit (catalog no. PK-6200; Vector Laboratories), according to the manufacturer's recommendations. The following antibodies were used for immunostaining: OC (1:5000), A-11 (1:1000), and anti-human p53 (1:2000).

For immunofluorescence sections using A-11, OC and p53, the same protocol used for BCC cells was performed. Other sections stained with conformational antibodies were then incubated with ApopTag Fluorescein in situ kit (Cat #S7110, Chemicon) to label apoptotic cells according to manufacture specifications.

### 2.8. Toxicity assays

*AlamarBlue assay:* SH-SY5Y cells and fibroblasts from basal cell carcinoma were grown in 96-well plates. Cells were treated with Aβ, α-synuclein and p53 monomers, oligomers or fibrils (10 μM) or with 1X PBS for untreated controls. All measurements were performed in triplicate. Cytotoxicity was measured using an Alamar-Blue assay kit (Serotec). Cell images were acquired using a EVOSxl microscope from AMG. Statistical analyses were based on a Two-Way ANOVA test, performed using Origin-8 software (Origin Lab).

## 3. Results

### 3.1. P53 forms oligomers and amyloid fibrils that cannot bind to DNA

To characterize recombinant p53 oligomers and fibrils, we performed a dot blot assay using the conformation-specific A-11 antibody, which recognizes toxic oligomers formed by a variety of amyloidogenic proteins [17] and OC antibody, which recognizes amyloid fibrils independent of the aminoacidic sequences [18]. Recombinant p53 freshly dissolved in phosphate buffer (p53 monomer) and p53 fibrils are not recognized by the A-11 antibody. However, recombinant p53 oligomers exhibits A-11 immunoreactivity (Fig. 1A). In the case of the p53 fibril sample was only detected with the OC antibody, confirming the presence of fibrilar amyloids in the preparation. For further characterization, AFM was used to investigate the morphological features of the aggregates. In the sample positive for A-11, a homogenous population of spherical oligomers of p53 with an average size of 2.6 ± 0.2 nm (Fig. 1B) was present. In Fig. 1C it is possible to see fibrillar structures of p53 which were also positive for OC. The formation of fibrils was also monitored using Thio-T dye-binding assay [19], which confirmed the formation of p53 fibrils (Fig. 1D).

To investigate whether the formation of p53 oligomers and/or fibrils leads to a loss of DNA binding, we utilized an ELISA that detects p53 when bound to consensus duplex deoxyologonucleotide

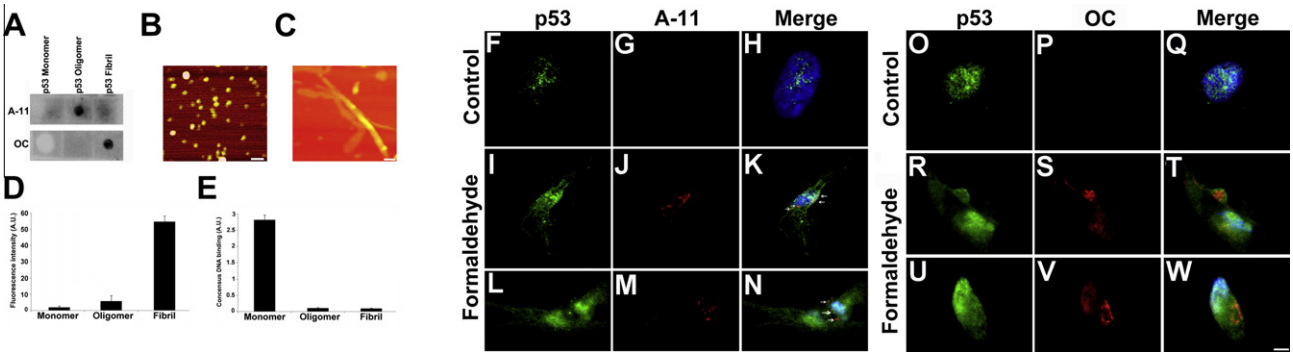


**Fig. 1.** Wild-type p53 forms oligomers and fibrils which cannot bind to DNA. (A) p53 oligomers were detected with A-11 and OC specifically detected the fibrillar conformation of p53. (B) p53 spherical oligomers and (C) amyloid fibrils were seen under AFM (Scale bar 60 nm for oligomers and 50 for fibrils). (D) p53 fibrils enhance thioflavin T fluorescence. Data are reposted as the mean fluorescence emission. (E) Graph of functional p53, demonstrated that after the aggregation of p53 to oligomers and fibrils, the protein losses ability to bind to DNA. (F, G and O–Q) BCC cells without the addition of formaldehyde show only intranuclear p53. Double staining with p53 and A-11 in BCC cells treated with formaldehyde revealed the presence of cytoplasmic p53 oligomers (white arrows) in the vicinity of the nucleus (I–N). Co-Staining with p53 and OC in treated cells demonstrated the formation of cytoplasmic p53 amyloid fibrils inclusions (R–W). Nuclei were stained with DAPI. Scale bar 15 μm.

sequences. Thus the ELISA is a primary readout of DNA-bound p53 and does not detect p53 that is not bound to DNA. In Fig. 1E it is possible to see that p53 oligomers and fibrils lost the ability to bind to DNA, demonstrating that the aggregation and amyloid formation produces a loss of function of p53.

### 3.2. Formation of p53 oligomers and fibrils in human Basal Cell Carcinoma cell culture

Studies have demonstrated that formaldehyde induces neuronal tau and Aβ to aggregate forming neurotoxic amyloids [20]. To investigate the formation of p53 amyloid aggregates in a cell-based system, we treated basal cell carcinoma cells with a low amount of formaldehyde for 72 h. A homogenous nuclear distribution of p53 was observed in untreated cells, without any presence of oligomers or amyloid fibrils (Fig. 1F–H and O–Q). In cells treated with formaldehyde and double stained with A-11 and anti-p53 antibody, a marked punctuate staining was observed, which accumulated throughout the perinucleus, and corresponded to p53 oligomers (Fig. 1I–N). In the case of the cells treated with formaldehyde and double stained with OC and anti-p53 antibody, large inclusions or aggregosomes of fibrillar p53 were detected in the cytoplasm (Fig. 1R–W). These results show that an inflammatory agent can directly or indirectly induce the misfolding and redistribution of p53 from the nucleus to the cytoplasm and alter p53 binding to DNA. Amyloid species from unidentified proteins were also observed in BCC cells treated with formaldehyde, therefore demonstrating that inflammatory agents also induce the malfunction of several proteins.

### 3.3. Detection of p53 oligomers and fibrils in human cases of basal cell carcinoma

To investigate the presence and distribution of amyloid in skin samples taken from 6 patients with BCC, sections were stained with Thioflavin S (Fig 2 C and D), and Congo Red (Fig 3 E and F). To confirm the presence of BCC, skin section were stained with H&E. Fig. 2A, control, displays a healthy skin section stained with H&E and Fig. 2B shows the distinct presence of tumor islands

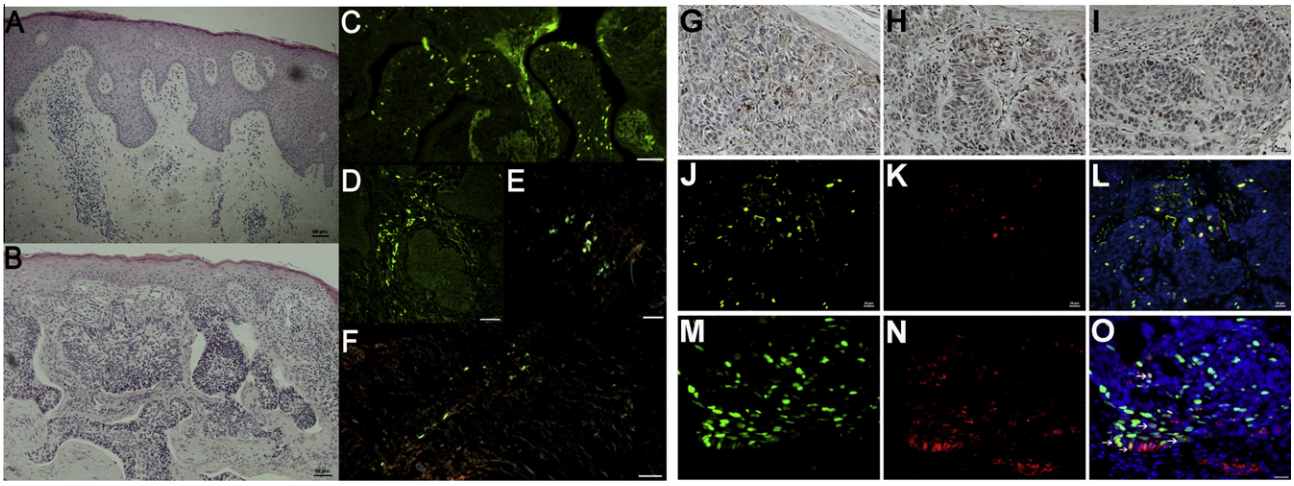


**Fig. 2.** Demonstration of amyloid deposits in human cases of basal cell carcinoma. (A) H&E staining demonstrate the differences between a normal skin and a (B) BCC case. In cases of BCC, it is possible to visualize the presence of tumor islands and cellular peripheral palisading in the basal layer. (C and D) ThioS staining of a representative case of BCC: green fluorescent aggregates were located in the stroma between the tumor islands and intracellularly in the tumoral cells. (E and F) The same amyloid pattern was detected with Congo red. Scale bar, 60 μm for control case and 50 μm for BCC cases. (G) OC stained skin sections of BCC were positive in the stroma between the tumor islands and intracellularly in the tumoral cells. (H) Positive oligomers were also located in the stroma between the tumor islands and intracellularly in the tumoral cells. (I) Staining revealed the accumulation of p53 in tumoral cells. (J and L) p53 fibril characterization using anti-p53 antibody and OC revealed a considerable amount of p53 deposits (green channel). The presence of amyloid fibrils is evidenced by a strong staining pattern with OC (red channel). The merged panel (L) shows a few areas of colocalization between OC and p53. (M and O) Skin sections were double immunostained with anti-p53 antibody (green channel) and A-11 (red channel) confirming the presence of intracellular p53 oligomers (white arrow). Nuclei were stained with DAPI. Scale bar 20 μm.

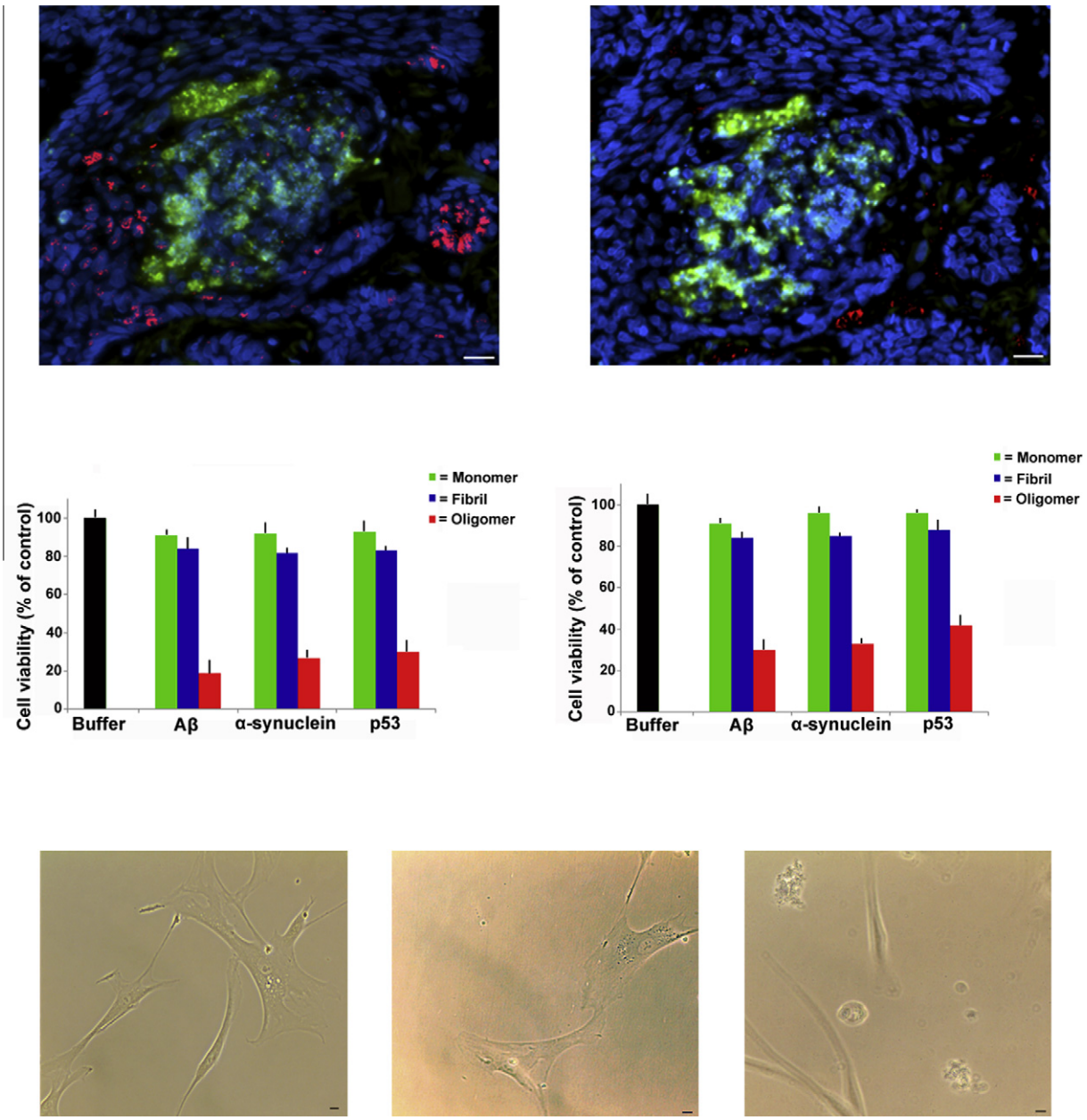


**Fig. 3.** Oligomers of p53 compromise cell viability. (A) Double staining with Apotag kit (green fluorescence) and A-11 (red fluorescence) demonstrated that oligomers are in the vicinity of apoptotic cells in cases of BCC. (B) Scant amyloid fibrils were detected with OC antibody (red fluorescence) associated with apoptotic cells (green fluorescences). Scale bar 10 μm. (C and D) Oligomers were toxic to cultured SH-SY5Y (C) and BCC cells (D). Oligomeric forms of Aβ, α-synuclein and p53, but not monomer or fibrils, demonstrated toxicity. (E and F) BCC cells treated with monomeric (E) and fibrillar (F) p53 did not show any morphological damage. (G) In contrast, a high level of cellular death was observed in BCC cells culture treated with oligomeric p53 (black arrows).

and cellular peripheral palisading in the basal layer, highly characteristic of BCC. The aggregates observed using Thio-S and Congo Red, were located in the stroma between the tumor islands, in the overlying subepidermis, and intracellularly in the tumoral cells.

We used OC and A-11 to demonstrate the presence of oligomers and fibrils in BCC (Fig 2G and H respectively). Fibrillar amyloids were detected in the stroma between the tumor islands and intracellularly in the tumoral cells (Fig. 2G). Oligomeric aggregates were also detected in the stroma between the tumor islands, but their localization was mainly inside malignant cells (Fig. 2H). We also performed staining using an anti-p53 antibody (Fig. 2I) and it is apparent that p53 accumulates exclusively in the cells forming the tumor.

Double staining was performed to examine the distribution of p53 amyloid species. Strong p53 reactivity was observed in the tumoral cells, but there was only partial overlap with fibrils positive for OC (Fig. 2J–L). Fibrillar signal showed only partial localization with p53 specifically intracellularly; non-co-staining material likely is constituted of other proteins involved in fibril formation, given its protein and structural heterogeneity [21]. Strong p53 immunoreactivity co-localized with A-11, revealing the presence of p53 oligomers in tumoral cells. Similar to results obtained using the fibrillar antibody, considerable A-11 signal was observed that failed to co-localize with p53 (Fig. 2M–O). Taken together, these results demonstrate the multi-faceted nature of amyloid aggregates in Basal Cell Carcinoma.

### 3.4. P53 oligomers promote cell death in basal cell carcinoma

We sought to examine whether oligomers correlate with cellular toxicity in BCC human samples. To accomplish this we performed immunofluorescence staining using the conformational antibodies A-11 or OC with the ApopTag in situ apoptosis detection Kit. As expected, oligomers, detected with A-11, were in the vicinity of apoptotic cells (Fig. 3A), but almost no fibrils were detected in areas rich in apoptotic cells (Fig. 3B). We also treated SH-SY5Y neuroblastoma (Fig. 3C) and BCC cells (Fig. 3D) with monomeric, fibrillar or oligomeric p53. Cells were also treated with monomeric, fibrillar or oligomeric Aβ or α-synuclein, which are highly amyloidogenic proteins involved in Alzheimer's and Parkinson's disease respectively. The cytotoxicity assay in both cell types confirmed that the oligomeric form of p53 is highly toxic, but the monomeric and the fibrillar forms were not (Fig. 3E–G). The same was observed for Aβ and α-synuclein, which has been previously reported [22]. Additionally, the toxicity presented by p53 oligomers show the contrasting possibility that with respect to this variant of skin cancer, there is not only a loss of function of p53, but also a gain of toxic amyloid function responsible for cellular demise in tumors.

## 4. Discussion

The present study, show how p53 can adopt different amyloid conformations, such as oligomers. Oligomeric structures composed of amyloidogenic proteins are thought to contribute to the pathogenesis of amyloid diseases [7]. The detection of p53 oligomers by A-11 and the morphology of these oligomers detected by AFM demonstrates that this amyloid species of p53 presents a similar amyloidogenic structure as other oligomers [21]. In addition to the recognition of fibrils with OC, p53 fibrils were characterized by AFM and Thio-T, further demonstrating the structural similarities with other fibrils previously described [21].

This is the first study where a homogenous population of p53 oligomers and fibrils are prepared without cross contamination between amyloid species, making it possible to analyze p53 misfolding and functionality at a specific level of aggregation. In relation to the loss of p53 function secondary to amyloid formation, a recent study has shown how cholesterol secosterol aldehydes induce amyloidogenesis of wild-type p53 protein and subsequently inhibits p53 biding to DNA [23]. In the present study, we show not only that p53 amyloid fibrils cannot bind to DNA, but also reveal the oligomeric form of the protein. This finding has great relevance if we take this new information into consideration in order to identify the presence of amyloid deposits in BCC samples, since reagents that are commonly used for this type of diagnosis, namely Thioflavin and Congo red, are insufficient to detect amyloid oligomers [19].

When the misfolding of p53 was induced in BCC cell, oligomers were observed in the vicinity of the nucleus and the formation of larger aggregosomes of p53 fibrils were observed in the cytoplasm. These results correlated with previous studies from Kaganovich and colleagues, where two intracellular compartments for the sequestration of misfolded proteins were identified [24]. The authors show that the partition of quality control substrates to either compartment seems to depend on their aggregation state. Specifically, soluble misfolded proteins, such as oligomers, accumulate in a juxtanuclear compartment where proteasomes are concentrated. In contrast, terminally aggregated proteins, such as fibrils, are sequestered in a perivacuolar inclusion [24]. These observations demonstrated that the misfolding of p53 at the cellular level, presented in this study, behave like any other protein misfolding process related with amyloidogenic diseases.

In BCC cells and human cases of the disease, we observed the presence of amyloid species of different protein origins other than p53. This may possibly be explained by a seeding and/or cross-seeding mechanism. This mechanism refers when pre-existent amyloid aggregates induce the aggregation of homologous (seeding) or heterologous proteins (cross-seeding) [25]. Our data, and that of other studies, support the idea that p53 not only is involved in tumor genesis by a loss of the antitumor function of the wild-type p53, but also by a gain of function that increases the aggregation potential of the misfolded mutant or wild-type p53 protein. Once present, the p53 aggregates can propagate the structural alteration to other functional correctly-folded p53 molecules in a prion-like mechanism, as already suggested [26]. In the case of the human samples, it cannot be determined if p53 misfolding triggers aggregation or promotes the amyloid formation of other proteins detected.

A recent study shows that p53 oligomers affect SH-SY5Y cell viability [12]. This was the first study to suggest the relevance of oligomers in p53 amyloid formation and exhibits a common pathogenic mechanism with amyloid diseases. In our study we demonstrated that p53 oligomers are highly toxic in comparison with p53 monomers and fibrils. We not only prove the toxicity of p53 oligomers in cell culture, but also we demonstrated how oligomers are in the vicinity of apoptotic cells in human cases of BCC, suggesting that this amyloid specie induce and precede cell death. This has been the first study to show, in human cancer samples, such as BCC, the relation of oligomers with cell death; a concept that has been extensively probed in several other amyloid diseases [7].

In conclusion, this study illustrates how p53 can adopt diverse amyloid conformations and that these amyloid species induce the loss of function and gain of toxic function of p53 *in vitro* (Fig. 4). These results suggest that cancer may be considered an aggregation-associated disease. In the majority of amyloid related diseases, the gain of toxic function of the amyloid protein is the relevant event in the disease pathogenesis, rather than the loss of function of the protein [7]. On the contrary the results shown here demonstrate that the loss of function of p53 is a more relevant event in the pathogenesis of BCC, rather the gain of toxic amyloid

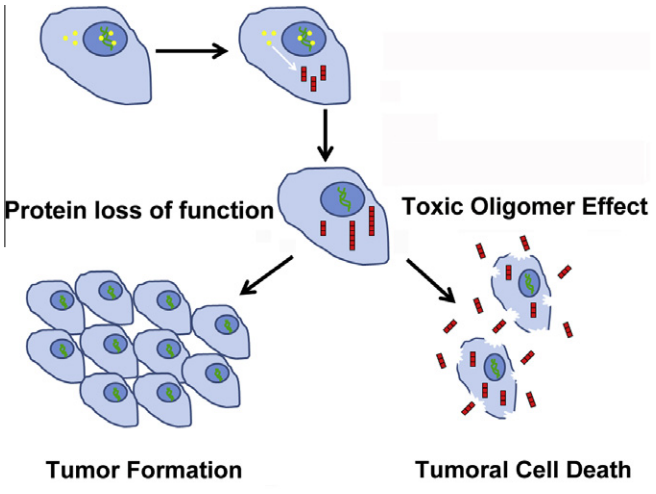


**Fig. 4.** Dual role of p53 amyloid formation in cancer. Wild-type p53 in normal conditions is a mediator of cell cycle control and maintenance of genome integrity and is located in the nucleus. In cancer cases, certain unknown factors could induce the misfolding of p53 into an amyloidogenic protein form, acquiring a loss of function translating into the inability to bind to consensus DNA sequences or a gain of toxic function, both characteristics of the amyloidogenic forms of certain proteins. In the case of p53 misfolding in cancer, the effects of a loss of protein function related with tumor progression has much more relevance than a gain of toxic function.

function. Nevertheless the important role of the protein aggregation process in both types of diseases, suggests that cancer treatment may be approached similarly as has been reported for other amyloid diseases.

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