Expanding the prion concept to cancer biology: dominant-negative effect of aggregates of mutant p53 tumour suppressor

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Synopsis

p53 is a key protein that participates in cell-cycle control, and its malfunction can lead to cancer. This tumour suppressor protein has three main domains; the N-terminal transactivation domain, the CTD (C-terminal domain) and the core domain (p53C) that constitutes the sequence-specific DBD (DNA-binding region). Most p53 mutations related to cancer development are found in the DBD. Aggregation of p53 into amyloid oligomers and fibrils has been shown. Moreover, amyloid aggregates of both the mutant and WT (wild-type) forms of p53 were detected in tumour tissues. We propose that if p53 aggregation occurred, it would be a crucial aspect of cancer development, as p53 would lose its WT functions in an aggregated state. Mutant p53 can also exert a dominant-negative regulatory effect on WT p53. Herein, we discuss the dominant-negative effect in light of p53 aggregation and the fact that amyloid-like mutant p53 can convert WT p53 into more aggregated species, leading into gain of function in addition to the loss of tumour suppressor function. In summary, the results obtained in the last decade indicate that cancer may have characteristics in common with amyloidogenic and prion diseases.

Key words: amyloid, cancer, p53, prions, protein aggregation, protein misfolding.

INTRODUCTION

p53 is a tetrameric nuclear phosphoprotein that plays an essential role in the prevention of cancer development by inducing cell-cycle arrest or apoptosis in response to a variety of stress signals, such as DNA damage. Disruption of the p53 network usually has severe consequences that favour cell survival and tumour progression [1–3]. p53 is regulated primarily by the ubiquitin ligase Mdm2 (murine double minute 2), which binds p53 and targets it for degradation in proteasomes [4].

The human p53 protein comprises 393 amino acid residues and three main functional regions: the N-terminal activation domain, which is able to interact with a variety of proteins; the CTD, responsible for tetramerization; and the core domain (p53C) comprising residues 94–312 that constitute the sequence-specific DBD (DNA-binding region) of the protein [5,6] (Figure 1). More than 90% of the point mutations in p53 that are related to malignancy are found in this segment [7].

Mutations in the p53 gene (TP53) are frequently associated with increased susceptibility to cancer development. Inactivation of p53-regulated pathways has been described in over 50% of all human cancers, making them interesting targets for cancer therapies [1,7–10]. Most of these mutations result in the expression of a p53 protein that has lost its WT (wild-type) functions, gained additional functions and may exert a dominant-negative regulation over the WT p53 [11]. Mutant p53 can acquire oncogenic functions, independent of WT p53, to drive mechanisms of cell migration, invasion and metastasis [11,12]; these events will be addressed below.

Abbreviations used: AD, Alzheimer’s disease; AFM, atomic force microscopy; ALS, amyotrophic lateral sclerosis; APR, aggregation-prone region; CTD, C-terminal domain; DBD, DNA-binding domain; HSP, heat-shock protein; Mdm2, murine double minute 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; PMD, protein misfolding disease; WT, wild-type.

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In addition to the loss of protein function caused by somatic mutations, aggregation of p53 in cells can also inactivate the WT protein, leading to malignancy [10]. Several cancer cells exhibit abnormal accumulation of WT or mutant p53 either in the cytoplasm or in the nucleus. This accumulation has already been described in neuroblastoma, retinoblastoma, breast and colon cancer cells [13–15]. There is evidence that the three functional domains of p53 could form amyloid-like aggregates [10,16–20], which leads us to speculate that p53 amyloid formation might participate in the malignant process [10,16].

The p53 protein has been extensively studied at both structural and functional levels. In this review, we outline how ordered and amyloid-like aggregates of the mutant p53 tumour suppressor might play a role in cancer development. We also discuss the pathways through which misfolded p53 could divert native proteins into aggregates, and how the mutant form, with its greater propensity for aggregation, would lead to a dominant-negative effect. These findings may reveal the biological significance of the prion-like behaviour of oncogenic p53 mutants and help to develop new strategies to disrupt the formation of aggregates. This approach would be particularly attractive for possible therapeutic applications in cancer and other PMDs (protein misfolding diseases).

**TP53 mutations and mutant p53s**

Mutations in *TP53* are very frequent in cancer and it is now clear that their occurrence is associated with bad prognosis, especially in breast cancer [21,22]. As a master regulator, p53 is at the centre of a node of complex pathways involved in cellular stress responses, including the maintenance of genomic integrity; based on these functions, Lane coined p53 as the guardian of the genome [23]. Inactivation of p53 leads to genomic instability and other hallmark features of cancer cells, such as resistance to apoptosis [24,25]. However, this is not the full story. In addition to the suppression of p53 activity, the presence of mutations in *TP53* may change the protein functionality to a dominant-negative form, sequestering WT p53 and other proteins or acquiring new oncogenic roles [26]. When p53 was discovered in 1979, it was described as an oncogenic protein associated with SV40 (simian virus 40)-transformed cells. Later, it was demonstrated that a mutant form, not WT p53, acted as a tumour suppressor. The discrepancies of the initial discoveries showing oncogenic or anti-oncogenic activities of p53 were later linked to the presence of either mutant or WT p53 forms in the different models. The results obtained in the past reinforce the observation that some mutant p53 proteins accumulate and have oncogenic roles during carcinogenesis [26,27]. For example, in 1979, De Leo and collaborators detected expression of a new cancer-related antigen, murine p53, in chemically induced sarcomas in mice; this protein was apparently absent in normal non-transformed cells, such as adult mouse fibroblasts, lymphoid cells, or haematopoietic cells [28]. A few years later, it was reported that the p53 'oncogene' had the effect of immortalizing cells, which was increased by mutational events [29]. A common feature of these first reports was the observation of p53 accumulation in cells and its interaction with viral proteins [26,27].

The human *TP53* gene [OMIM (Online Mendelian Inheritance in Man) 191170] is located at 17p13.1 and is composed of 11 exons (Figure 2). It has two transcription start sites in exon 1 and alternative splicing sites in intron 2 and between exons 9 and 10. Moreover, an internal promoter and transcription start site was described in intron 4 [30,31]. A common ancestral gene is believed to have existed in primitive organisms and then duplicated during evolution resulting in a family of genes composed of *TP53*, *TP63* and *TP73*. p53-related proteins are present in species from worms to humans. In contrast with other tumour suppressors, *TP53* mutations are characteristically point mutations instead of complete or partial deletions, such as observed in *Rb* and *BRCA1* [32]. These mutations are very diverse in nature, and, in human cancers, they are predominantly located in exons 5–8, which correspond to the p53 DBD (Figure 2). In fact, a number of these mutations are informative about the mutagenic agent involved in the process of carcinogenesis. Such association...

Interestingly, only TP53 mutations that induce significant structural and functional changes in p53 will generate cell proliferative advantage and genomic instability, resulting in biological selection [26]. Such biological selection is intrinsically associated to the natural history of tumours, and the presence of mutant p53 is frequently linked to poor patient prognosis. Recently, Shah et al. showed that TP53 is the most frequently mutated gene in tumours of triple negative (ER−, PR− and HER2−) breast cancer [36], considered an aggressive class of breast cancer. In a study dedicated to correlating the loss-of-function mutations in TP53 with breast cancer prognosis, the authors found that mutations are more frequent in high-grade, large-size, node-positive cases and also in oestrogen- and progesterone-receptor-negative tumours [22]. Additionally, the different mutations were not equal in relation to their associated clinicopathological characteristics; for example, p.R248W was associated with poorer prognoses [22]. Therefore at least in breast cancer, mounting evidence suggests that TP53 mutations act by driving oncogenic aggressiveness and different mutations are not equivalent in this process [37].

In human cancers, the most common p53 mutants are derived from missense somatic mutations at ‘hot-spot’ residues located at the DBD. Some of the most frequent mutations include R175H, a structural mutant, and R248Q, R273H, R248W, R273C and R282W, which are contact mutants that have misfunctional interactions with the target DNA [32]. It is currently known that mutant p53s are characteristically stable and accumulate in cancer cells. This accumulation, however, is not direct proof of the presence of mutant p53 because WT p53 may also accumulate. Additionally, null p53 mutants do not accumulate, so the absence of accumulation is not direct proof of the lack of p53 mutations [38]. Altered protein stability and accumulation is of key importance to the aggregation state and, consequently, to the dominant-negative effect [10,19,20].

In general, p53 mutants may be classified according to their functional alterations: neutral, negative, dominant-negative or gain-of-function [32]. The last two classes are of great interest because they may participate in cancer genesis and evolution [11].

Gain-of-function of mutant p53 in cancer cells was well established by Di Como et al. in 1999 [39]. The authors demonstrated that both p.R175H and p.R248W decrease the ability of p73 to promote apoptosis. More recently, Muller et al. 2009 showed that overexpressed mutant p53 promotes invasion and metastasis through inhibition of p63 [40]. Evidence on the role of p63 in modulating EGFR (epidermal growth factor receptor)/integrin signalling and the association between mutant p53 and increased cell migration both in vivo and in vitro were also previously presented [40].

An interesting example of the connection between mutant p53s and cancer development is their role in the control of miRNA (microRNA) expression [41]. Ectopic expression of mutant p53s (R273H, R175H and C135Y) through stable transfection into the p53 knocked-down HEC-50 cell line, a cell line derived from endometrial cancer, showed that these mutants repressed the expression of miR-130b and triggered ZEB1-dependent epithelial–mesenchymal transition and cancer cell invasion [42]. Thus, mutant p53 may interact with diverse gene promoters in addition to its known interactions with different proteins. Such activities illustrate the flexibility and multifaceted functions of p53 variants.

PMDs and the prion concept
PMDs are usually attributed to proteins that are able to convert their native structure into β-sheet rich aggregates under certain circumstances. PMDs are characterized by the presence of a combination of mature fibrils, protofibrils and oligomers, which accumulate in the intra- or the extracellular environment of affected tissues [43–45] (Figure 3A). This wide group of PMDs includes several neurodegenerative diseases, such as AD (Alzheimer’s disease), Parkinson’s diseases, prion diseases [also known as TSEs (transmissible spongiform encephalopathies) and ALS (amyotrophic lateral sclerosis)], among others. Although there is still a lot to learn about the mechanisms involved in these diseases, the growing number of maladies related to the protein misfolding phenomenon has given rise to an increasing knowledge on the subject.

Protein misfolding can occur at many different stages between the synthesis of a new protein and its degradation. Protein misfolding occurs due to gene mutations, which lead to an inability of the produced protein to correctly fold, malfunction of the protein trafficking machinery and abnormal protein interaction with different molecular partners in the cellular milieu [10,44,46,47]. The aggregates can be formed intracellularly, as is the case with α-synuclein (Parkinson’s disease), SOD (superoxide dismutase) (in ALS) and huntingtin (Huntington’s disease) [43] or extracellularly, as is the case in prion diseases [48,49]. Type 2 diabetes [due to the aggregation of the IAPP (islet amyloid polypeptide)] [50,51] and AD [43].

The pathogenic role of these protein aggregates has long been discussed. Although large aggregates are the morphological markers of these diseases, they appear to be less toxic than oligomers, which display strong toxicity when incubated with cultured cells or inoculated into animals [52-54]. Oligomers usually progress in size and shape to form protofibrils and fibrils [55] (Figures 3A and 3B). Protein misfolding, accompanied by an increase in β-sheets, is generally associated with amyloid fibril formation, as they have a common cross-β scaffold [55,56]. A
with the discovery that several other proteins, such as Aβ (amyloid β-peptide), tau and α-synuclein, can behave as prions, demonstrated through their transmissibility using either mammalian cell cultures or animals [63–66]. In vivo evidence has been found in patients with Parkinson’s disease submitted to transplant of fetal mesencephalic dopaminergic neurons, in which the formation of α-synuclein-positive Lewy bodies was detected in grafted cells [67]. These findings lead to a wider interpretation of the prion concept and may lead to the inclusion of other amyloid diseases in the group of prion diseases. Based on this concept, the next topic will focus on the misfolding and aggregation of p53 and its relation to protein malfunction and cancer.

p53 misfolding and aggregation: amyloids and the prion-like behaviour

Formation of high-molecular-mass species of p53 was described in the early 1990s [68]. By this time, p53 was already known to be related to cell-malignant transformations, although it was believed that the aggregated form of p53 was a quaternary structure produced to prevent its rapid degradation. It was observed that p53 forms high-molecular mass aggregates through self-aggregation or through interaction with various cellular and viral proteins [69,70].

The instability of p53 has been the subject of several biophysical studies, with denaturation being reached by chemical, thermal and pressurization means [16,71–74]. Our group demonstrated the formation of different types of aggregates (fibrillar, annular or granular) after physical-induction of unfolding of the p53 central core domain (p53C) [16,73]. Mild denaturation of p53C by pressure generated fibrillar aggregates (Figure 4), which were characterized by AFM (atomic force microscopy) and binding to thioflavin T was observed [16] (Figures 4A, 4B and 4F). Pressure treatment also produced annular aggregates that were observed immediately after decompression (Figure 4E); such aggregates were toxic to cells in culture, as shown by an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] reduction assay (Figure 4C). Heat denaturation generated granular-shaped aggregates (Figure 4D) that were more cytotoxic than the pressure-induced aggregates [16] (Figure 4C).

The central core domain of p53 is the main target for mutations; more than 90% of hot-spot mutations in p53 occur in p53C [7,32]. We have shown that hot-spot mutants have a greater tendency to aggregate than WT p53 (Figure 5) [20,73]. Using different techniques, such as X-ray diffraction, electron microscopy, FTIR (Fourier-transform infrared) spectroscopy, DLS (dynamical light scattering), cell viability assay and anti-amyloid immunoassay, we demonstrated the amyloid nature of the aggregates (Figure 6) [20].

Apart from the central core domain, the transactivation and tetramerization domains have also been shown to form amyloid aggregates [17,18,75]. It was shown that unlike the WT p53 tetramerization domain, the G334V mutant forms amyloid fibrils by a two-step process under physiological temperature and pH conditions [75]. Additionally, this mutant was capable of forming heterotetramers with WT p53. Aggregate formation and protein conformational changes were evaluated by CD, binding of thioflavin T, AFM and Congo red birefringence [75]. The transactivation domain (residues 1–63), when exposed to low pH,
formed aggregates enriched in β-sheets, presenting an amyloid pattern as shown by thioflavin T-binding, AFM and X-ray diffraction [18]. Taken together, these findings suggested that the whole protein could aggregate into amyloid assemblies, which was later demonstrated by several groups [19,20,76,77].

We detected p53 aggregates in archived samples of breast cancer tissues expressing mutant R248Q and other p53 hot-spot mutants using co-localization assays with A11 and anti-p53 DO1 antibodies [20,76] (Figure 7). In general, there was a strong correlation between tumour aggressivity and p53 aggregation. In the case of breast tissue biopsy bearing R248Q, the patient had an invasive ductal carcinoma of Elston grade 3 [20].

We also found co-localization of full-length p53 and aggregates in tumour cell lines [20]. In the case of MDA-MB 231 cells (that express the R280K p53 mutant), there was massive aggregation in the cell nucleus [20] (Figure 7). When these aggregates were extracted from the cells, they eluted in the void volume of a gel-filtration column.

APRs (aggregation-prone regions) are found in most proteins. These small APRs are normally sheltered from aggregation due to intra-protein interactions or because they are hidden in the hydrophobic protein core. However, when the protein structure is disturbed by mutations or environmental changes, these sequences can be exposed, thus favouring protein aggregation [78]. One of these sequences has been detected within the p53 hydrophobic core of the DBD by Xu et al. [19]. This sequence is exposed when several mutations occur and leads to aggregation of p53. This aggregation inactivates not only the product of the WT allele of the TP53 gene but also other proteins belonging to the p53 family, p63 and p73, which have very similar aggregating-prone sequences that facilitate co-aggregation. Perinuclear aggregates formed after transfection with mutant p53 were detected through immunostaining and confocal microscopy. These results were confirmed in different types of tumours, such as human colon adenocarcinoma, mouse lung metastases from osteosarcoma and mouse kidney lymphoma [19]. This co-aggregation abrogates the p53 paralogous function, a consequence of the characteristic gain-of-function role of these mutants.

The formation and kinetics of WT and mutant p53 amyloid oligomers and fibrils has been widely discussed [20,79–82]. p53 aggregates have been detected in different types of cancer biopsies, such as breast cancer [20,76] and basal cell carcinoma [80]. Additionally, cholesterol secosterol aldehydes, which are lipid-derived aldehydes frequently detected in chronic inflammation, appear to play a role in the formation of p53 amyloid aggregates [77]. Moreover, p53 function appears to be lost during incubation with these compounds, thus showing a possible interplay between chronic inflammation and cancer development, which is often reported in epidemiological studies [77].

The DNA-binding properties of p53 are frequently lost upon p53 aggregation; however, the small cognate double-stranded DNA has been shown to stabilize both the p53C domain and full-length p53 and to have the potential to rescue aggregated and misfolded species [83] (Figure 8). Therefore, such DNA sequences could be exploited as a new approach to cancer therapy in the near future. The use of small aptameric nucleic acids and other polyanions has been proposed to modulate aggregation of the prion protein [47,62,84]. On the other hand, p53 has been previously described to form long fibrillar segments complexed with DNA [85].

The presence of intermediate states that participate in the p53 aggregation route has been highlighted in different studies [8,72,86]. Mutants are unstable and appear to unfold and aggregate faster than the WT form of p53 [20]. An intermediate conformation of p53C during equilibrium and kinetic folding/unfolding transitions induced by GdmCl (guanidinium chloride) has been demonstrated [74]. Moreover, experiments combining high hydrostatic pressure and sub-zero temperatures [73] were able to evidence an intermediate state with a fold similar to the hot-spot mutant, R248Q, as demonstrated by NMR. These data indicate a finely energetically regulated mechanism for the formation of p53 aggregates, which, at first glance, may appear random. New investigations in this area might provide a better characterization of this pathway. The regulation of this process is definitely
promising for the modulation of p53 aggregation in cancer cells.

The dominant-negative hypothesis for WT p53 inactivation in cells carrying a mutation in one of the alleles of the TP53 gene proposes that the presence of even a single mutant protein in the formation of the p53 tetramer would lead to loss-of-function of p53 [87,88]. Our group has previously proposed an alternative hypothesis for the dominant-negative effect in which WT p53 at lower concentrations would be incorporated into aggregates containing the mutant p53 (Figures 5B and 5C) [10,16]. In our hypothesis, the presence of a misfolded conformation of mutant p53 would sequester the properly folded form, abrogating its function, in a prion-like mechanism. We found that R248Q mutant oligomers and fibrils could seed aggregation of WT p53 [20] (Figure 5B). The lag phase of WT p53 aggregation was suppressed demonstrating the high seeding potential of the aggregated mutant protein.

As represented in the diagram (Figure 5C), mutant p53 would convert the WT form into a faster aggregating species [20]. Hetero-oligomerization would more likely occur in smaller aggregates, and the formation of fibrils would make aggregation an irreversible phenomenon. These findings are also in line with the results that the anti-oligomer antibody bound more targets in tumour tissues containing the p53 mutations [20,76].

A misfolded pathogenic species would act as a molecular chaperone inducing the correctly folded form of p53 to adopt a misfolded conformation, a seed that would then increase aggregation exponentially. The higher susceptibility to aggregation of mutant p53 would amplify this process [20]. We propose that the aggregated form of mutant p53 may act as a sink, sequestrating the
Prion-like behaviour of p53 may lead to cancer

Figure 6 WT and R248Q p53 aggregate into amyloid fibrils

The participation of other proteins on p53 aggregation and formation of intracellular aggregates and aggresomes
As mentioned before, misfolded forms of p53 can occur either due to mutations or to situations in which p53 is inactivated. As described here, the co-aggregation of p53 and its paralogues, p63 and p73, were demonstrated intracellularly [19]. The erroneous interaction of p53 with the chaperone Hsp70 (heat-shock protein 70) and their interaction with Mdm2 promoted the formation of misfolded aggregates of p53, giving rise to structures named ‘pseudo-aggregates’ with β-amyloid characteristics [89]. It was proposed that these interactions may stabilize p53 mutants in cells. Hsp90, on the other hand, appears to interact with p53 and convert it to a molten globule state [90], a conformation that is also observed when p53 is native protein into the inactive conformation through a mechanism described for a typical prionoid [63,64]. This reinforces the proposal that cancer should be considered, at least in part, as a prion disease [10, 20]. Finally, the possibility of modulation of this phenomenon [82,83] opens up new possible targets for cancer chemotherapy.

Figure 7 Tumour samples bear oligomeric species of WT and mutant p53
The prion-like behaviour of oncogenic p53 mutants, as represented in Figure 5(C), provides an explanation for the dominant-negative effect and the gain of function, including the high metastatic potential of cancers bearing p53 mutations. The inhibition of aggregation of p53 into oligomeric and fibrillar amyloids by nucleic acid aptamers and small molecules appears to be a good target for therapeutic intervention in tumour diseases.

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