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review

p53 - the paradigm of tumor-suppressor genes?

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p53 is a tumor-suppressor gene the alterations of which are among the most frequent genetic changes detected in human neoplasms. Its product - p53 protein is a component of several biochemical pathways that are central to carcinogenesis: DNA transcription, genomic stability, DNA repair, cell cycle control, and apoptosis. The analysis of the spectrum of p53 mutations and insight into the p53 mediated biochemical pathways of programmed cell death and cell cycle arrest, provide clues to the understanding of molecular pathogenesis of cancer and of mechanisms related to p53 mediated tumor suppression. The purpose of the present article is to summarise the most important facts concerning p53 since understanding of the above listed processes might provide the potential molecular targets for the development of a rational cancer treatment.

Key words: neoplasms; genes, suppressor, tumor; genes, p53; protein p53

Historical background

In 1979, Lane and Crawford,[1] as well as Linzer and Levine[2] independently discovered p53 as a nuclear 53kd phosphoprotein tightly associated with the large T antigen in the SV40 tumor virus-transformed cells. Originally, p53 protein came to be classified as a tumor antigen since it was suggested that the interaction of p53 with the large T antigen was important for transformation.[1,2] The p53 cDNA constructs isolated in this period were all derived from tumor cells[3] and were found to cooperate with the ras oncogene to transform rat fibroblasts in cell culture.[4,5] So, p53 came to be classified as an oncogene. Finally, in late 1980s, all the transforming p53 cDNA clones were discovered to be mutant forms of p53, while the wild-type gene isolated from normal cells failed to induce neoplastic transformation and even inhibited tumor cell growth or blocked the neoplastic transformation.[6-10] Now p53 looks like being a tumor-suppressor gene, negatively regulating the cell cycle and requiring loss-of-function mutations for tumor formation. However, unlike other classical tumor-suppressor genes, at least some mutated p53 forms act as dominant transforming oncogenes.[11]

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Structure and regulation

The p53 gene spans a moderately-sized segment of DNA, located on the short arm (17p13) of chromosome 17 and is ultimately

translated to a phosphoprotein consisting of 393 amino acids contained in 11 exons, the first of which is noncoding.[12] Five evolutionary conserved domains within the coding regions are supposed to be essential to the functional activity of p53.[12,13]

The N-terminal domain (residues 1-42) interacts with the subunits of the general transcription factors TFIID and TFIIH and acts as a transcriptional activator. This domain also binds the MDM-2 protein - a negative regulator of p53, and adenovirus E1B protein. The core domain (residues 100-300) harbors the sequence specific dsDNA binding function of p53, and encodes the binding site for SV40 large T antigen and, possibly, for the papillomavirus E6 protein. The C terminal domain (residues 300-393) has multiple functional activities, including nonspecific DNA binding and reannealing of complementary ssDNA oligonucleotides. Residues 320 to 355 are involved in oligomerization, and the very terminal C domain (residues 360-393) binds ssDNA ends and regulates specific DNA binding by the core domain.[14-18]

It appears that p53 alone assembles into inactive forms and requires activating factors to confer an effective sequence-specific DNA binding capacity. Such a regulation is exerted by the C-terminal end of p53 itself. Hupp *et al.* proposed a model according to which the C-terminus negatively regulated specific DNA binding by interacting with a region in another p53 molecule within the tetramer.[19] This locked the tetramer in a conformation that was incapable of specific DNA binding.

p53 contains multiple phosphorylation sites located at both the C and N-termini of the molecule. Eight different protein kinases are involved in p53 phosphorylation: p34cdc2 kinase, DNA-activated protein kinase, mitogen activated protein kinases, protein kinase C, casein kinase I and II, Raf-1 kinase, and Jun kinase.[20-26] p34cdc2 kinase (an A- and B-cyclin dependent kinase) phosphorylates at serine 315 and thus stimulates the specific binding of DNA to the consensus sequence of p53 and also causes a specific conformational change of the protein.[20] DNA-activated protein kinase and mitogen activated protein kinases are involved in the phosphorylation of p53 at the N terminal domain, influencing the transcriptional activity and the half-life of the protein.[21,22] Protein kinase C-dependent, direct or indirect, phosphorylation of serine residues 372-381 at the C terminal of the p53 tetramer is a critical event for the transition from the latent to the active form of p53. Namely, the "open" configuration of the four phosphorylated C ends of the tetramer is a necessary prerequisite for the nonspecific DNA binding which, in turn, allows the consequent specific DNA binding to p53 consensus motifs.[19,23,27] The phosphorylation of the serine 392 is dependent on casein kinase II, however, this site is less critical for p53 activation.[28]

Another form of p53 regulation is exerted at the level of p53 protein stability. In normal cells, p53 shows a relatively short half-life (about 20 minutes) due to its rapid turnover, yet its half-life can be extended to hours following some kinds of cellular stress or as a consequence of mutations involving the core domain. Stability of the protein is affected by its complex formation with a number of cell proteins that are capable of slowing-down or preventing its ubiquitin-pathway degradation.[29,30]

It is still uncertain which physiological signal activates p53 after an appropriate stimulus. Possible candidates are p300 and the closely related transcription factor CBP that bind to N-terminal domain of p53. p300 acetylates conserved lysine residues in the p53 C-terminal domain which results in the activation of specific DNA binding of p53.[31,32]

Intracellular localisation, concentration, and state of phosphorylation of p53 are cell-cycle dependent. Activity of wild-type p53

protein demands nuclear localisation of the protein which occurs close to the beginning of S phase. Following the beginning of DNA synthesis, p53 accumulates again in the cytoplasm.[33,34]

Function

p53 protein is implicated in nearly all forms of cell growth stimulation and inhibition. It may be required early in the induction of cell proliferation and is also a transcriptional regulatory protein, capable of both stimulating and repressing gene expression.[35] p53 binds DNA in a sequence-specific manner and also influences gene expression indirectly by interacting with other transcription factors.[35,36] In certain cell types, over-expression of p53 induces apoptosis.[23,37,38] p53 may regulate in vitro cellular senescence and, under the influence of certain cytokines, it cooperates in the induction of differentiation.[39,40]

Transcription dependent pathway

Several genes were found to be transcriptionally activated by p53, including MDM-2, p21, GADD45, cyclin G1, BAX, FAS, transforming growth factor-alpha, muscle kreatinine kinase, and insulin-like growth factor-binding protein 3.[10,41-48]

Following DNA damage, p53 protein rapidly accumulates in the nucleus. The C-terminal domain of p53 recognises the damaged DNA, and the accumulation of p53 is probably a consequence of conformational change of the protein which leads to reduced degradation by ubiquitin degradation pathway or, less likely, a consequence of increased synthesis of p53 protein.[33,38,49] At the same time, no changes in p53 mRNA levels are observed.[50]

The p53 protein, in turn, activates downstream genes whose products are involved in growth inhibition, e.g. p21 and GADD45. p21 is a cyclin-dependent kinase inhibitor that inhibits the activity of cyclin D-cdk 4/6 causing a hypophosphorylation of retinoblastoma protein (Rb), thus preventing the release of E2F and blocking the G1-S transition.[51,52] Transactivation of GADD45, the protein product of which binds proliferating cell nuclear antigen and inhibits S phase entry, may contribute to the p53 dependent cell cycle arrest pathway.[53] Insulin-like growth factor-binding protein 3 gene which encodes a protein that binds insulin-like growth factor and thus inhibits its growth signalling is another p53 target gene that may function in this pathway.[48] p53 also regulates the G2/M checkpoint of the cell cycle, yet the mechanism of p53 mediated G2/M control is unknown.[54,55]

The expression of MDM-2 protein is regulated by the level of wild-type p53 protein. The MDM-2 protein, in turn, forms a complex with p53 and decreases its ability to act as a positive transcription factor - which represents a negative feedback loop to buffer changes in p53 levels.[56,57]

Transcription independent pathway

Modulation of cellular processes goes often via the mechanism of protein-protein interactions. In agreement with its multifunctional qualities, p53 protein associates with a group of viral and cellular proteins that may play an important role in the p53 mediated and transcription independent pathway (Table 1).[35,36]

Several basic transcription factors, including TATA binding protein, TATA binding protein-associated proteins, TFIIH-associated factor p62 form a complex with p53.[58-61] Binding of TATA binding protein to p53 protein has been implicated to be responsible for p53 mediated transcriptional repression. The list of genes reported to be transrepressed by p53 consists of proliferating cell nuclear antigen, interleukin 6, Rb gene, multidrug-resitance (MDR) gene, p53, BCL-2, inducible

Table 1. Some of viral and cellural proteins that associate with p53

Viral proteins	Cellular proteins
human papilloma virus E6	heat-shock protein 70
simian virus 40 T antigen	MDM-2
Epstein-Barr nuclear antigen	ubiquitin-ligase E6-AP
adenovirus E1B	transcription factor WT-1

nitric oxide synthase-2.[62-67] The binding of p53 to replication protein A also alludes to the possible direct role of p53 in DNA replication and nucleotide excision repair.[68]

p53 in the nucleotide excision DNA repair

The observations that p53 can selectively bind to several DNA helicases, including XPB and XPD, which are a part of transcription factor TFIIH, led to the hypothesis that p53 may play a direct role in modulating DNA nucleotide excision repair.[69,70] Furthermore, p53 can also recognise several forms of damaged DNA (mismatched DNA, ssDNA ends).[71] So, a new model emerged in which p53 may act as a sensor that binds to damaged parts and recruits the nucleotide excision repair machinery by trapping TFIIH (i.e. the major component of the repair complex) at regions where it is needed which, in turn, facilitates the constitution of a functional "repairosome".[72]

p53 mediated apoptosis

The molecular mechanisms behind p53 induced apoptosis are only partially explained. The current idea is that DNA damage induces stabilization of the p53 protein which promotes DNA repair by assembling the repair machinery.[70,71] In case the DNA damage is unrepairable, p53 triggers cells to undergo apoptotic death to prevent propagation of the cells carrying a mutation. Several activities of p53 have been identified that could participate in the process of programmed cell death. Namely, p53 upregulates the expression of BAX and downregulates expression of BCL-2, all of which have been implicated in modulation of apoptosis.[73] Another possible explanation for the induction of apoptosis could be that the transactivation of insulin-like growth factor-binding protein 3 gene and thus increased insulin-like growth factor-binding protein 3 levels may presumably block an insulin-like growth factor mediated survival signal and lead to apoptosis.[48] Finally, a whole series of new p53 induced genes related to redox control have been discovered that lead to the formation of reactive oxygen species, oxidative degradation of mitochondrial components and apoptotic cell death.[74] Beyond this, a transactivation independent function of p53 in the triggering of the apoptotic pathway has been implicated and may well be performed by a proline rich region located between residues 64 and 91 in p53 molecule. The proline rich region may provide a crucial accessory apoptotic signal, perhaps by interacting with a cellular SH3-domain-containing partner protein.[75,76]

Briefly, the major role of p53 is being a monitor of cellular proliferation (guardian of the genome) and a determinant of response to DNA damage.

Mutations

The p53 mutations are found in the preponderance of human tumors and the functional p53 is lost in approximately half of all human malignancies.[52,77]

The majority of p53 mutations are missense point mutations giving rise to single

amino acid substitutions that abrogate the specific DNA binding activity.[18] Concomitantly, the half-life of p53 extends from normal 20 minutes (wild-type protein) to approximately 48 hours (mutant protein) resulting in nuclear accumulation of the mutant protein.[78,79]

Most of the mutations are clustered in the most highly conserved domains of the gene spanned by four to nine exons. There are at least three mutation "hot spots" affecting the residues 175, 248, and 273.[80] Although mutations of the p53 gene are most frequently acquired, they can also be inherited through the Li-Fraumeni syndrome. In these families, one mutant p53 allele is inherited, and the second allele acquires a mutation.[81]

p53 is not inactivated only through mutation, but also at the protein level through complexing with DNA tumor viral oncoproteins like the SV40 large T antigen, the adenovirus E1B protein, and the human papilloma virus E6 protein[82] or cellular protein MDM-2.[57]

Detection of p53 mutations

In respect to the fact that inactivation of p53 in tumor cells leads to the increased cellular proliferation and inhibition of apoptosis and concerning the observations that mutations of p53 gene are associated with advanced disease, poor response to chemotherapy or radiotherapy, and short survival,[83,84] it is of great importance to determine the p53 status in every patient prior to treatment. Various approaches to the detection of p53 mutations have evolved in the last 19 years and each of them has certain advantages and certain disadvantages.[77,85]

The most informative method for the study of p53 mutations is the determination of the nucleotide sequence with either direct sequencing or indirect molecular analysis.[85] Molecular sequencing is the only way to evaluate the mutational event that inactivates the gene (there is no accumulation of the mutant protein) and allows for the unequivocal detection of alterations. On the other hand, the indirect molecular methods as denaturing gradient electrophoresis, single-strand conformational polymorphism analysis or variants as hydroxylamine and osmium tetroxyde chemical cleavage, and pulse field gel electrophoresis, are more suitable for screening and easier to perform. Yet, both methods share some drawbacks - namely, they cannot be at the moment performed in routine diagnosis, tumor tissue is required, and care must be taken to avoid contamination from an excess of normal tissue.[85-87]

Immunohistochemical and immunocytochemical methods, under optimum conditions, are capable of detecting most missense mutations (which result in nuclear accumulation of the mutant protein) and can also identify p53 stabilization without mutations (a consequence of the alteration of pathways regulating p53 expression). On the contrary, the mutations which do not induce p53 overexpression (nonsense mutations, frame-shift mutations, splice mutations, gene deletions, promoter mutations) will go undetected. Immunohistochemical results can be affected by the degradation of antigen during tissue processing and by the specificity of the antibodies used. Tumor tissue is needed, but the contamination by normal tissues is not a critical factor. In sum, p53 immunostaining is still an imperfect reflection of the prevalence of p53 mutations.[85,86,88-90]

It remains controversial whether the mutant p53 protein can be detected in patients sera, since the results of various determinations are opposing. Namely, two groups of authors determined the serum levels of mutant p53 in patients with malignant lymphomas using a commercially available ELISA kit,[91,92] while another group of authors failed to do so using the same ELISA method in patients with lung cancer.[87] Simi-

larly, Hassapoglidou using immunofluorimetric method could not detect mutated p53 protein in sera of patients with cancer.[93]

Although p53 is a cellularly encoded protein, it has been found to be immunogenic and capable of eliciting a p53 specific antibody immune response. About one third of patients (the percentage varies for different types of cancer) with tumors that carry p53 missense mutations develop circulating p53 antibodies. These antibodies are not seen if there is no p53 accumulation in the tumor cell and, in case of lung carcinoma, they can appear before the cancer is detectable. The p53 protein may either be released during tumor cell necrosis, or otherwise translocates to the surface of the cell, inducing a B-cell response as a result of the breakdown of the immune system tolerance. Methods used for the determination of p53 antibodies include ELISA, immunoblot, and immunoprecipitation techniques. These methods can be performed routinely, they do not require tumor tissue, and can be used for follow up. Therefore, assessment of serum p53 antibodies is quite specific, but has low sensitivity (some mutations do not induce the production of p53 antibodies) in the detection of p53 mutations.[85,87,94-99]

Therapeutic approaches

Several therapeutic approaches are currently being assessed against the growth advantage and resistance to chemotherapy and radiotherapy observed in tumor cells with p53 mutations. The first approach is the investigation of active immunization against the potential tumor antigens carried by mutated p53, and indeed, it has been shown that it is possible to generate p53 specific CD8+ cytotoxic T lymphocytes by immunizing mice with mutated p53 protein.[100] Furthermore, it was observed that a monoclonal antibody to p53, PAb 421, and a small peptide derived from p53 (the C-terminal domain) are able to restore the sequence specific DNA binding as well as growth suppression function of at least some mutant p53 proteins (by inducing a change in the configuration with a return to the active wild-type configuration).[19,101-103]

Among the recently proposed approaches, two are quite interesting. The first uses an adenovirus defective for E1B gene, which replicates only in the cells lacking functional p53 but not in the cells with wild-type p53, leading to selective destruction of tumor cells with mutant p53.[104] The second, on the other hand, utilizes the transfer of a cytotoxic gene which is only activated in the presence of a mutant p53, resulting in a selective killing of tumor cells with p53 mutation.[105]

However, the most promising approach is p53 gene transfer in tumor cells carrying a p53 mutation. In tumor cells lacking functional p53, such a transfer can lead to tumor regression, as well as improve the cytotoxicity of antineoplastic agents, and the response to ionizing radiation.[106-108] The most frequently used vectors for p53 gene transfer in animal models have been recombinant adenoviruses, and less often retroviruses, which have a lower capacity of gene transfer *in vivo*. Interestingly, some tumor regressions were more important than expected. They were indicated by the percentage of p53 transfected cells, suggesting a possible "bystander" effect, with destruction of non-transfected cells in the vicinity of transfected cells, as for suicide gene transfer.[109]

And finally, another idea was to try to identify the drugs that may trigger programmed cell death through a p53 independent pathway. It has been suggested that taxol could be one of them, however, the clinical results with taxol were poorer in patients with mutant p53.[110,111]

Latest findings

Even though p53 seems to play a central role in nearly all forms of cell growth stimulation and inhibition and was termed as the "guardian of the genome", it is becoming obvious that other proteins, as for example the recently discovered p33 and p75, also take an important part in the regulative mosaic.

The nuclear protein p33 (a product of the tumor-suppressor gene ING1) forms a complex with p53 and cooperates in the negative regulation of cell proliferation by modulating p53 dependent transcriptional activation.[112,113]

p73 is a protein that is closely related to p53, both structurally and functionally; however, it is induced by different signals and thus plays a fundamentally different role in the maintenance of cell homeostasis. It can, at least when overproduced, activate p53 responsive genes and act as a growth suppressor.[114,115]

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