*JKGUI 2003; 10 (Edisi Khusus): 406 - 415*
*Diterbitkan di Jakarta*

**Jurnal Kedokteran Gigi**
**Universitas Indonesia**
ISSN 0854-364X

# CELLULAR RESPONSES TO DNA DAMAGE AND ONCOGENESIS BY THE p53 AND pRb/E2F PATHWAYS

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**Auerkari EI, Suwelo IS, Tjarta A, Cornain S, Rahardjo TW, Eto K, Ikeda-MA:** Cellular responses to DNA Damage and Oncogenesis by the p53 and p Rb/E2F Pathways. Jurnal Kedokteran Gigi Universitas Indonesia. 2003; 10 (Edisi Khusus): 406-415

## Abstract

Cellular responses to stress including DNA damage show multiple options involving the mechanisms of growth arrest, DNA repair and programmed cell death or apoptosis. Failures in these mechanisms can result in oncogenesis or accelerated senescence. Much of the response is coordinated by p53, a nuclear phosphoprotein with a central role in the defences against physical, chemical and pathogenic agents which challenge the DNA integrity. The p53 pathways for mobilising the cellular defences are linked to the pRb/E2F pathways regulating the cell cycle progression. This paper aims to review the current understanding on the networks and main molecular machinery of these processes. In addition, the implications on cellular decision making for the defences as well as evolutionary aspects of these mechanisms are discussed in brief.

Key words: Cellular responses; p53 pathways; pRb/E2F pathways

## Introduction

### Responses to damage: cell cycle arrest, repair or apoptosis

The importance of the nuclear phosphoprotein p53 in the DNA maintenance is evident from the observation that the corresponding tumor suppressor gene is the most commonly altered gene in human cancer, with a mutation frequency in tumors of about 50%. Many tumors without p53 mutations involve either other mechanisms of p53 inactivation or alteration, or defects in the cellular pathways regulated by p53.[1,2] Germline mutations of p53, as in the Li-Fraumeni syndrome, are associated with an elevated risk developing a variety of cancers at an early age. In addition, murine models lacking functional p53 develop cancers at early age.[2] Unstressed cells contain only minor amounts of p53 protein, which is targeted by the protein MDM2 for nuclear transport and ubiquitin-mediated

proteolysis. Stress e.g. due to DNA damage can stabilise p53 by several mechanisms that can either make p53 resistant to MDM2 or inhibit it, leading to p53 accumulation and subsequent cellular defence reactions coordinated by p53. The p53 pathways mobilised in stressed cells are partially linked to the pRb (Retinoblastoma protein) and E2F pathways which regulate the cell cycle progression also in unstressed cells. When activated, pRb inhibits $G_1$/S transition and thereby cell proliferation by binding the E2F transcription factors. Alterations or loss of function in the pRb/E2F pathways are also frequently observed in tumors, even with fully functional (wild type) p53.[2,3]

Known agents of DNA damage include physical stimuli like UV light, X-ray and gamma irradiation, a wide array of chemical mutagens and carcinogens, including chemotherapeutic agents, and certain pathogens, e.g. *Helicobacter pylori* (gastric cancer), human papillomavirus (HPV, cervical cancer), and hepatitis B and C viruses (HBV, HCV, liver cancers), and probably many more remain to be discovered and characterised. The damage can result in a particularly severe tendency towards carcinogenesis if the damage extends to the very part of the genome which is required for maintaining the DNA integrity. In addition to external agents, host susceptibility is a contributing factor in carcinogenesis, and this susceptibility can be hereditary or acquired. However, with intact defensive pathways, DNA damage and other cellular stress factors such as oncogene activation, hypoxia or heat shock will invoke mobilisation of an array of reactions (Figs 1 to 3).

When the damage is not too extensive and does not cripple the DNA repair machinery, the chances are that the damage can be repaired. Apart from actual mechanisms of DNA repair, this will require some time without cell prolifiration, i.e. cell cycle arrest. In case of extensive, irrepairable damage, the protective machinery can induce apoptosis or programmed cell death of the compromised cells. The p53 gene and the corresponding protein have a central role in the processes of cell cycle arrest and apoptosis through mechanisms which are clearly of great importance in protecting the cells from carcinogenesis. The protective function of p53 is lost if it itself is mutated or inactivated, or if the cascade of the proteins which p53 regulates to deal with DNA damage is impaired.[4,5] Possible damage in this machinery includes (Fig 1) alteration in e.g. cyclin-dependent kinases (Cdk), their inhibitors (Cdi), cyclins and the pRb family of proteins, sets of which are involved in the transition from $G_1$ to S phase and from $G_2$ to mitosis.[5,6]

Reasonably modest damage to DNA can be repaired by mechanisms which include nucleotide excision repair, base excision repair, mismatch repair, reversal of simple alkylation adducts by $O^6$-methylguanine DNA methyltransferase, and in severe cases with no original template, by recombination.[7] Although p53 controls the DNA damage checkpoint, the DNA repair processes appear at least partially independent of p53. Nevertheless, the p53-coordinated arrest of the cell cycle provides the essential time for this repair to prevent the damage from spreading to the next generation. In addition, there is evidence that p53 participates at least in base excision repair, and may be involved in other repair processes that require proofreading.[5] The repair processes are not infallible, and some fraction of the damage can remain either because repair fails, is not complete or because some error is reintroduced during repair or in subsequent replication (Fig 2). As in the case of original damage, the resulting defects may be tolerable, accelerate senescence or lead to neoplastic growth.

To stop the cell cycle after detected DNA damage (Fig 3a), a set of mechanisms operates to arrest the $G_1$ phase, preventing the cell from entering the S phase. There are several inhibitor proteins for $G_1$ arrest, such as $p27^{Kip1}$ and $p16^{INK4a}$, of which for example the latter can induce arrest mediated together by pRb and the related proteins p130 and p107.[6] One of the

main routes for $G_1$ arrest after DNA damage are initiated by p53, although also nonfunctional $p16^{INK4a}$ regardless of the p53 status may appear in cancer cells. $G_1$ arrest accounts for 15-40% of arrested cells after DNA damage, and is mainly mediated by $p21^{Waf1/Cip1}$, a cyclin-dependent kinase inhibitor which is transcriptionally activated by p53 after DNA

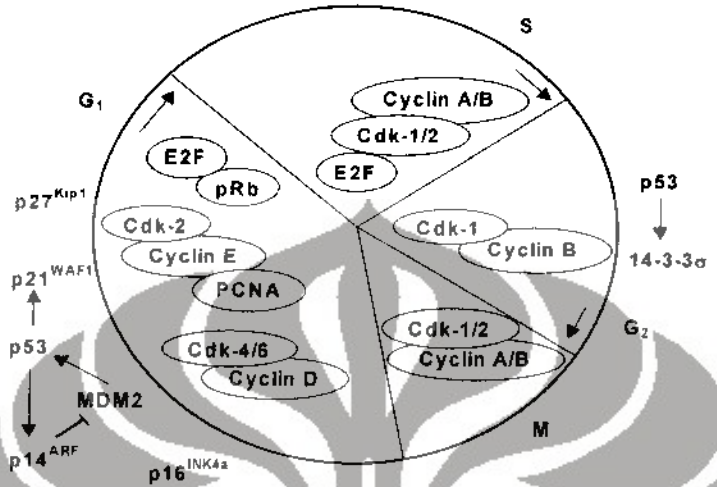


Fig 1. A simple model of cell cycle, including Cdk-cyclin complexes and some Cdk inhibitors that contribute to arrest of the cycle. For example, as cells enter the cycle from $G_0$ phase (not shown), cyclin D associates with its catalytic partners Cdk-4/6 from about mid-$G_1$ phase, and cyclin E with Cdk-2 late in $G_1$. Of the opposing Cdk inhibitors, $p16^{INK4a}$ inhibits Cdk-4/6, and $p21^{Waf1}$ and $p27^{Kip1}$ also Cdk-2. The pRb family of proteins form complexes with the E2F transcription factors to repress $G_1$ to S transition, while free E2F will promote it. Parallel processes can arrest $G_2$.

damage. Also other activators including E2F1 can induce $p21^{Waf1/Cip1}$.[8,9]

The second set of mechanisms after DNA damage will arrest the cell cycle in the $G_2$ phase, preventing the cell from initiating mitosis. This type of arrest is mediated among others by 14-3-3σ, which is activated through p53 dependent regulation. The 14-3-3σ protein is a cyclin-dependent kinase inhibitor, as is apparently another protein GADD45. The resulting block of the cell cycle in $G_2$ accounts for 60-85% of the arrested cells after DNA damage.[8,10]

In case of sufficiently severe DNA damage or failed repair, p53 can coordinate the cell to apoptosis (Fig 3b). Apoptotic process is mediated by a variety of signals, and a well established protein promoting apoptosis is Bax, which is upregulated by p53. Bax promotes release of cytochrome c from mitochondria, leading to a cytotoxic caspase cascade and eventually to cell death. Bax is counteracted in this by Bcl-2, which is repressed by p53. Other apoptosis promoters which are also upregulated by p53 include PERP, PML, Fas/APO1, Killer/DR5, the PIG family of genes, PAG608 and IGF-Bp3, but their functional mechanisms are less well known.[10,11,12]

The decisions on the balance of cell cycle arrest, DNA repair and apoptosis are based on the detected cellular stress and damage, and on the status of the mobilised defences (Figs 2 and 3). Different cell types can show considerable differences in these responses, so that for example while p53 is generally important in invoking the defences, at least in head and neck squamous cell carcinoma (HNSCC) apoptosis after irradiation and chemotherapeutic treatment can be independent of p53.[13] In general, carcinogenesis can result when the defences fail either because they show some specific weakness or simply by chance given sufficient time and accumulated damage.

Damage can also accelerate cellular aging or senescence, which limits the number of cell cycles and the proliferating life span of most

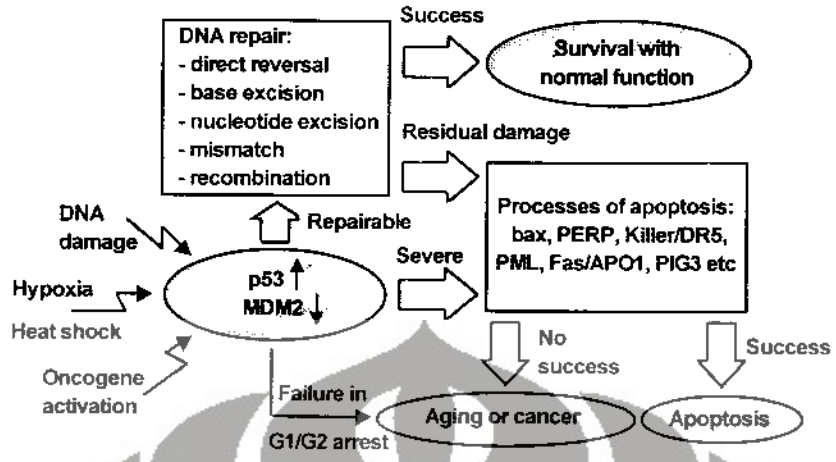


Fig 2. Main routes where failure in cellular defences to DNA damage can lead to carcinogenesis. Note that even in case of survival with normal function, the cells remain subject to aging (senescence) and the background rate of additional damage.

Table 1. Some important regulatory components in the cell cycle and defence systems against DNA damage, with main functions and chromosomal locations.

| Gene/ expression | Main function | Chromosomal mapping |
|---|---|---|
| p53 | Damage checkpoint, arrest/apoptosis | 17p13.1 |
| $p14^{ARF}$ | Binds MDM2 to stabilise p53 and arrest $G_1$ | 9p21-22 [1] |
| $p16^{INK4a}$ | Induces $G_1$ arrest mediated by pRb, p130, p107 | 9p21-22 [2] |
| $p21^{Waf1}$ | Inhibits Cdk-cyclin, arrest of $G_1$ | 6p21.2 |
| $p27^{Kip1}$ | Inhibits Cdk-2, arrest of $G_1$ | 12p12 - 12p13.1 |
| p130 | Retards $G_0$ to $G_1$ transition with E2F4 | 16q12.2-13 |
| pRb | Retards $G_1$ to S transition with E2F | 13q14.2 |
| Bcl-2 | Inhibits apoptosis (counteracts Bax) | 18q21 |
| ATM | Signals DNA damage to p53 / MDM2 | 11q22 |
| BRCA1 | Promotes apoptosis, can activate $p21^{Waf1}$ | 17q21 |
| Cyclin D | Control of $G_1$ to S transition (with Cdk-4,6) | 11q13 |
| E2FBP1 | Stimulates E2F transcription, promotes $p21^{Waf1}$ | 19p13 |
| 14-3-3σ | Inhibits Cdk-cyclin, arrest of $G_2$ | 1p35 |
| Killer/DR5 | Promotes apoptosis | 8p21 |
| PERP | Promotes apoptosis | 6q24 |

1) reading exons 1β, 2 and 3; 2) alternative reading exons 1α, 2 and 3

cell lines in multicellular eukaryotes, and can be seen as an additional protective mechanism against accumulating damage.

Some of the essential regulatory genes/proteins of the cell cycle and their chromosomal mapping are summarised in Table 1. This list is only a sample of the growing set of functional molecules involved in the cellular responses to damage, growth regulation and oncogenesis.

## E2F and related proteins in the cell cycle progression and arrest

The E2F family of proteins are transcription factors required for cell cycle progression and thereby also in oncogenesis.

In these processes the p53 pathway and pRb/E2F pathway appear to cooperate in determining the outcome of DNA damage.

E2F1 (and probably E2F2, E2F3 and E2F4) binding to pRb, as well as in parallel E2F4 or E2F5 binding to p130 (or p107) inhibits transition from $G_1$ to S.[6] Also, $p16^{INK4a}$ can inactivate the cyclin D-dependent kinases Cdk-4 and Cdk-6 that are needed for phosphorylation of pRb to mediate passage through G1/S. Dephosphorylation of pRb (and p130/p107) can release E2F, which are needed to express the S phase regulatory genes. Furthermore, E2F proteins can transactivate $p21^{Waf1/Cip1}$ transcription, leading to $G_1$ arrest.[7,9,15]

Loss of pRb function leads to loss of $G_1$ arrest and can result in apoptosis after DNA damage. Simultaneously increasing amount of free E2F promotes the progression of the cell cycle. In this way loss of pRb is equivalent in outcome to overexpression of E2F. Inversely, overexpression of pRb can also block p53-dependent apoptosis.[13] Independent of its apoptotic function, Bcl-2 can increase $p27^{Kip1}$ and p130 levels to maintain complexes of p130 and E2F4. These complexes retard the transition from $G_0$ to $G_1$, possibly by delaying E2F1 expression.[15]

E2F1 is a cell cycle promoter, but also a potential oncogenic signal inducing $p14^{ARF}$, which can bind MDM2 and thereby stabilise p53 and promote apoptosis in presence of combined increasing levels of $p14^{ARF}$ and E2F1.[10] The E2F1 protein contains a basic helix-loop-helix (bHLH) hydrophobic zipper structure required for DNA binding and dimerization in the central section, and a transactivation domain in the C-terminal part of the molecule.[16]

E2F activity is generated by formation of heterodimers between E2F1 (or one of the related proteins E2F2 to E2F5 ) and DP1 (or one of the related proteins DP2 or DP3). The resulting heterodimers have greater DNA binding activity than any of the homodimers, which in this way act synergistically in E2F site-dependent transcriptional activation. The E2F site-dependent transcription, in cooperation with E2F1, is stimulated by E2FBP1, a protein with a HLH motif but lacking the basic and zipper structures found around HLH in E2F1.[16] The corresponding gene coding for E2FBP1, also called *DRIL1*, is a homolog of the murine *bright* and Drosophila *dead ringer* genes, which however have different functions.[17]

E2FBP1 has been recently been shown to be directly regulated by p53. Ectopic expression of E2FBP1 activates $p21^{Waf1/Cip1}$ in cooperation with p53, inducing growth arrest which fails in cells deficient in functional p53.[18]

This process features therefore yet another link between the pRb/E2F dependent growth regulation and the p53 dependent pathways. These pathways can be interfered in oncogenesis by intercepting any of the principal regulating factors on the p53 to $p21^{Waf1/Cip1}$ axis, or alternatively on the $p16^{INK4a}$ to pRb axis, including the cyclin dependent kinases, and various inhibitors and activators within these systems. The interdepencencies of these interwoven systems are characterised by simplified models shown in Fig 3.

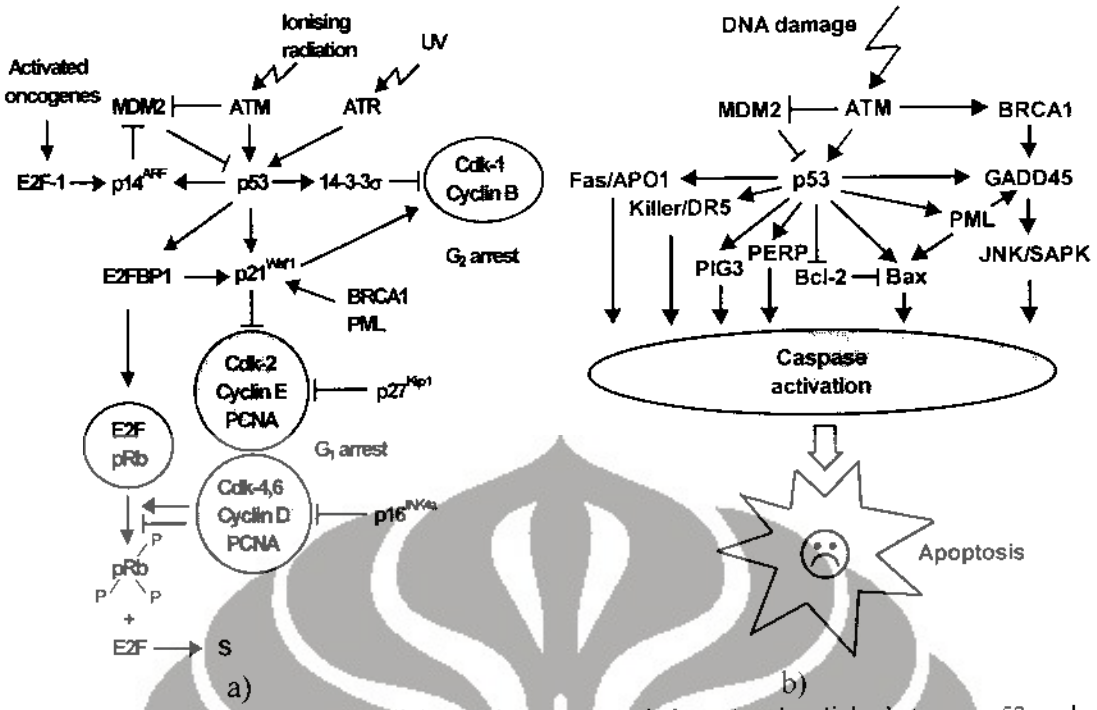


Fig 3. Simple models of a) the cellular cycle arrest regulation, showing links between p53 and pRb/E2F pathways; and b) apoptosis mainly including p53-dependent pathways. An arrow signifies activation/promotion or process direction, a line with a terminal bar inhibition. Note that several of the indicated regulatory molecules appear in reality as a family of proteins with mostly parallel functions, e.g. the INK family of kinase inhibitors includes $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$.

## Discussion

It is notable that the same type and extent of DNA damage can lead to DNA repair in some cell types while inducing apoptosis in others.[5] In principle this could reflect cell specific differences either in sensitivity to apoptosis or in the relative intensities of the repair and apoptotic mechanisms (or both), but the actual mechanisms are largely obscure. Cell type related differences are known for example of the p53 family members p73 and p63, which tend to be restricted to certain tissues. However, the only member of the family with undisputed role in the cellular defences is p53, which is ubiquitously expressed in all cells.[19]

If p53-mediated repair and cell cycle arrest functions were very essential for maintaining the integrity of the genome, p53 and its functions could be expected to be widely conserved in evolution.[5] Although p53 appears in mammals with typical conserved transcriptionally active regions, no p53 even in primitive form has been found in prokaryotes or in most groups of simple eukaryotes. A p53 homolog does occur in *Drosophila*, but does not have a role in cell cycle arrest, only in apoptosis which may be the original function of p53. There are proteins (such as E2F1 and BRCA1) which can transactivate $p21^{Waf1/Cip1}$ in p53-independent fashion[9], and other features of the cellular defences which are better evolutionarily conserved than p53. Several essential molecules in the cellular defences appear to be related to systems of growth, development and differentiation. This is not only true from comparison to non-human homologs but also within human cells. For example the regulatory proteins p73 and p63, which probably are related to the ancestor of p53, are active mainly during embryonal development rather than in tumor suppression.[19,20]

To evolve, the p53-dependent defences of complex eukaryotic organisms therefore must have had selective advantage which is weaker or not present in simple organisms. It is conceivable that the complexity itself in the cellular regulation systems could provide this advantage, because increasing complexity provides an increasing number of attack points for oncogenic damage. Another reason could be related to longevity of organisms. With longer life of an individual, which tends to be roughly related to the body size, metabolic rate and other features of physiology, there is an increasing chance of accumulating significant damage during the reproductive period, and therefore presumably some selective pressure for improved defences. Further comparisons of the p53 homolog functions within vertebrates and invertebrates is likely to clarify the issue.

The selective advantage aspect extends to pathogens inducing DNA damage. For example, it is an advantage to viruses to inactivate p53 or its function for replicating their own genome. E.g. HBV which encodes a protein (HBXAg) binding to p53 and preventing its transactivation, is thought to be involved in the pathogenesis of 90% of hepatocarcinomas.[3] Similarly, the HCV core protein can repress basal and induced $p21^{Waf1/Cip1}$ transcription and therefore intercept the p53-regulated cell cycle arrest.[9] Pathogens typically have several parallel mechanisms to disarm the host defences, and can also challenge other critical points of the regulatory system like the pRb/E2F pathway.[3]

Also carcinogenesis requires selective advantage for proliferation of neoplastic cells. These cells must overcome the defences and like with the pathogens, this can be done in a variety of ways, as is reflected in the differences between different types of cancers and tumors. However, there are a few common features for nearly all cancers. Excluding rare special cases, like retinoblastoma (embryonal eye cancer due to loss of pRb), epidemiological studies suggest that on average about six (with extremes of 3 to 12) oncogenic events are required to develop cancer.[21] The classic analysis for this purpose has assumed that the rate determining event of carcinogenesis is the neoplastic transformation of a single initiating cell. The assumption is not consistent with observed *in vivo* mutation rates or cancer incidence in humans with hereditary oncogenic defects.[22] The discrepancy is explained if the rate determining event is assumed to involve many (up to thousands) of cells, and indeed intercellular signalling via EGFR and other factors between cells, and cell group events such as angiogenesis are now seen essential for the growth of most tumors.[21,23]

Thus there are important differences between initial oncogenic events related to the defences of a singular cell, and actual development of tumors. The latter must be seen as a dynamic process of simultaneous proliferation, growth coordination and apoptosis of initiated cell groups. For example, while apoptosis is an essential defensive mechanism after severe DNA damage, increasing rate of apoptosis in tumors is generally a sign of advanced stage and poor prognosis rather than the opposite.[23] Although induced apoptosis by irradiation and chemotherapeutic agents is applied in cancer therapy, the resulting additional survival time is not always very long.[24] This partly reflects the disadvantage from compromised genomic defences, and partly the selective advantage for resistant cells that can adapt to the treatment and proliferate. In principle it were an advantage to fortify the defences instead of only mitigating the consequences, and new cancer treatments to this effect are being tested.[24] From the application point of view, this is obviously one of the driving forces for the current research into the cellular defences against DNA damage.

The mammalian genome has high reactivity and may undergo over 100 modifications a day. This is significant attack in spite of the large size of the genome- about 3 billion base pairs, of which perhaps 4% is coding - since the modifications and their consequences are

not random. Classic examples of carcinogenic signatures in p53 are hot spot codon mutations, like UV-related C→T and CC→TT conversions, aflatoxin B-induced G→T changes, tobacco-related G→T and G→C mutations, and A→T and T→A alterations associated with vinyl chloride.[25] In general, alteration or loss of expression in tumors concentrate on regulatory factors like p53, pRb, $p16^{INK4a}$, $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and other tumor suppressor factors in the networks of the cellular defences.[3,4,6,9,13,15] Nevertheless, even with such defects and subsequently increased cancer risk, many individuals predisposed to defects in these regulatory factors will not suffer carcinogenesis. Apart from the time and exposure to damaging agents required to accumulate the necessary number of oncogenic events, this probably also reflects the redundancies of the networks of the defence systems. Much needs to be clarified in the functional mechanisms of these networks and their molecular components, of which many remain yet to be found and characterised.

Table 2. List of abbreviations.

| Abbreviation | Meaning |
|---|---|
| ARF | Alternative reading frame |
| ATM | Ataxia telangiectasia mutated (gene) |
| ATR | ATM-Rad3-related (gene) |
| Bax | Bcl-2 antagonist-X |
| Bcl-2 | B-cell lymphoma/leukemia - 2 (gene) |
| Cdk | Cyclin-dependent kinase |
| Cdi (or Cki) | Cyclin-dependent kinase inhibitor |
| EGFR | Epidermal growth factor receptor |
| E2FBP1 | E2F binding protein 1 |
| GADD45 | Growth arrest and DNA damage 45 (gene) |
| HBV / HCV | Hepatitis B / C virus |
| HPV | Human papilloma virus |
| INK | Inhibitor of cyclin-dependent kinase |
| JNK | c-jun N-terminal kinase |
| MDM | Murine double minutes (gene) |
| p | Protein (or chromosome short arm) |
| PERP | p53 apoptosis effector related to PMP-22 |
| PCNA | Proliferating cell nuclear antigen |
| PIG | p53-induced gene |
| pRb | Retinoblastoma protein |
| q | Chromosome long arm |
| UV | Ultraviolet |

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