The Dual Role Played by p21 May Influence the Apoptotic or Anti-Apoptotic Fate in Cancer

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Abstract: p21 is a cyclin-dependent kinase inhibitor that is activated in response to different stress stimuli and could act as cell cycle suppressor. p21 can bind and inhibit cyclin-dependent kinase/cyclin complexes to mediate growth arrest in G1 and G2 phases. This condition enables DNA repair and suggests that p21 could have a role of tumour suppressor. p21 is one of the transcriptional targets of p53, a protein up-regulated after cellular stress stimuli. Besides the classical p53-dependent activation, p21 transcription can be achieved by other regulators as Sp1, STAT and AP2 in a p53-independent manner. Depending on cell type and cellular conditions p21 can have anti-apoptotic or pro-apoptotic functions being involved either in tumourigenesis or in tumour suppression. The function exerted is based on subcellular localization. In the nucleus p21 inhibits proliferation by blocking the cyclin dependent kinases while in the cytoplasm it acts inhibiting pro-apoptotic protein determining cell death inhibition. The different subcellular localization is related to different prognostic role of p21 in cancer and the cellular context in which it is expressed determines if it can be considered as a specific therapeutic target or as a marker of poor prognosis. This review focuses on the recent understanding of the functions of p21 with particular attention to the dual role detected in cancer where p21 can act as tumour suppressor promoting apoptosis or as oncogene preventing it.

Keywords: p21, Tumourigenesis, Cell cycle arrest, Apoptosis, Subcellular localization.

INTRODUCTION

Tumourigenesis is determined by the unbalance between cell proliferation and apoptosis. In cancer, the lack of apoptosis is related to the aberrant regulation of cyclin-dependent kinases (CDKs) and their cognate inhibitors (CKIs), whose binding negatively regulates cell cycle.

p21, a well-known protein acting as a cell cycle arrest inductor, was the first to be discovered and the most studied member of cyclin-dependent kinase inhibitor family. Its expression is usually induced by p53 protein after DNA damage, and it has a role in inhibiting several cyclin dependent kinases (CDK2, CDK3, CDK4 and CDK6) resulting in G1 cell cycle arrest and in block of transition in S phase [1]. p21 is also able to determine cell cycle arrest in G2 phase [2], through its interaction with PCNA (Proliferating Cell Nuclear Antigen), an essential cofactor for DNA polymerases [3].

Differential expression of p21 is involved either in tumourigenesis or in tumour suppression. Loss or mutation of p21 represents the main genetic alteration in cancer because of its frequent association with carcinogenesis increase [4, 5]. Furthermore p21 knockout determines spontaneous tumour growth in mice [6] while in some tumours p21 seems to prevent apoptosis [7].

In the light of more recent studies, p21 can exert an opposite role being anti-oncogenic or oncogenic, depending on the cancer type and on the drug treatment. In this context, by its complex and controversial role, the understanding of p21 functions becomes fundamental. In fact p21 has a different prognostic role in several cancers and, according to the cellular context in which it is expressed, it can have a dual role being a target of specific therapies, or a marker of poor prognosis. In this regard great attention has been given to its subcellular localization since p21 has been associated to the cell cycle arrest when present in the nucleus and to the apoptotic block when localised in the cytoplasm.

p21 localization can be strongly influenced by post-translation modifications, such as specific phosphorylation events on threonine and serine residues (Thr 145, Ser153, Ser 146, Ser 160 and Ser 130). These phosphorylations are mediated by different protein kinases that acting as intracellular signalling, promote p21 stabilization and loss of its specific function in term of negative cell cycle regulator. In fact phosphorylation inhibits the interaction of p21 with CDK/cyclin complexes or with PCNA. In particular, Thr 145 and Ser153 phosphorylations relocalize p21 in cytoplasm while Ser146, Ser160 and Ser130 do not

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allow its translocation from nucleus to cytoplasm. Despite all these modifications, Thr 57 phosphorylation does not have any evident effects on the inhibition of CDKs/cyclin in G1, while it is possible to evidence cell cycle arrest in G2/M phase transition.

Until now the pro-apoptotic function of p21 has not been deeply studied yet. Taking into account that defects of cell cycle arrest and apoptosis could be responsible for tumourigenesis and resistance to cancer therapies, it is important to elucidate the p21 pro-apoptotic mode of action.

In this review we will discuss about the current knowledge of the p21 dual role, pro- or anti-apoptotic, that may have various effects in different tumours describing recent data correlating p21 cellular localization to its function.

p21 AND CELL CYCLE

p21 belongs to the CIP/KIP (CDK Interacting Protein/Kinase Inhibitor Protein) family of cyclin-dependent kinase inhibitors (CKIs) that includes also p27 and p57. These proteins were identified for their ability to regulate cell cycle by inactivating Cyclin-Dependent Kinases (CDKs), key regulators of the cell cycle.

In DNA damaged cells, p21 plays a key role determining cell cycle arrest in G1 phase thus preventing the replication of damaged DNA (Figure **1**).

During cell cycle the phosphorylation of Rb protein (Retinoblastoma protein) along the G1 phase, is exerted by the CDK2/CyclinE complex and leads to the release of the E2F transcription factor, which activates genes needed for S phase. Rb is a tumour suppressor protein that when dephosphorylated does not release E2F so it avoids the cell cycle progression inhibiting E2F activity (Figure **2**). p21 directly inactivates CDK2 blocking the transition from G1 to S phase. It is also able to interact with CDK2/cyclinE and CDK2/cyclinA complexes binding specific sites. The binding occurs through two cyclin binding sites located at the N-terminus and at the C-terminus. The first motif (Cy1) has a high affinity for cyclin A binding, while the second one (Cy2) binds the cyclin B with low affinity. The N-terminal region also contains a specific CDK2 kinase-binding site [8]. The binding of p21 to CDK2 prevents enzymatic modifications in CDK2 as a specific activating phosphorylation (Thr160) or dephosphorylation (Thr14 and Tyr15), suggesting that p21 could compete with these enzymes for this binding [9].

The transition to S phase and the DNA replication can be inhibited by other p21 direct associations either to E2F, producing its inactivation [10, 11], or to PCNA, a cofactor of DNA polymerase δ and ε, essential for DNA synthesis and repair [3].

p21 is able to influence G2/M checkpoint interfering with CDK1/cyclinB that must be activated for the mitosis transition. p21 can block this process binding

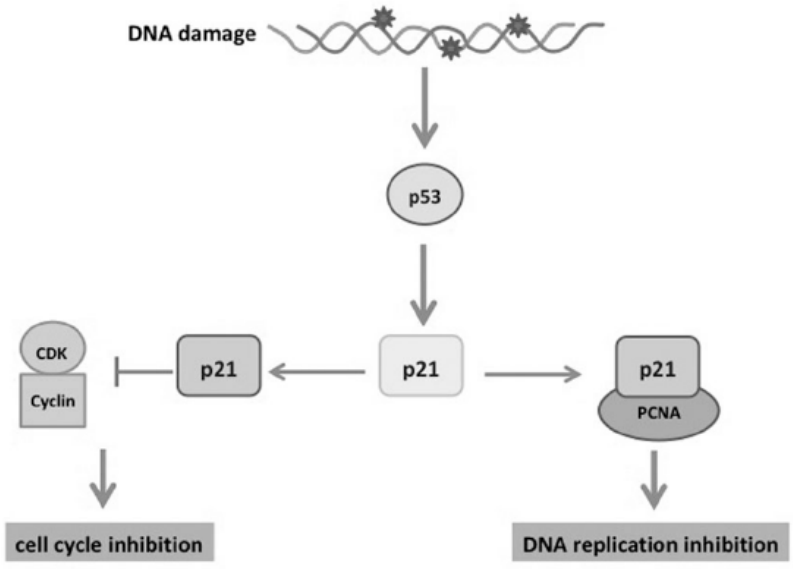

Figure 1: An example of the inhibitory effect of p21 on cell cycle progression. DNA damage increases p53 levels that activates p21 transcription. p21 inhibits cell cycle progression binding CDK/cyclin complexes or blocks DNA replication through the interaction with PCNA, a cofactor of DNA polymerase δ and ε.

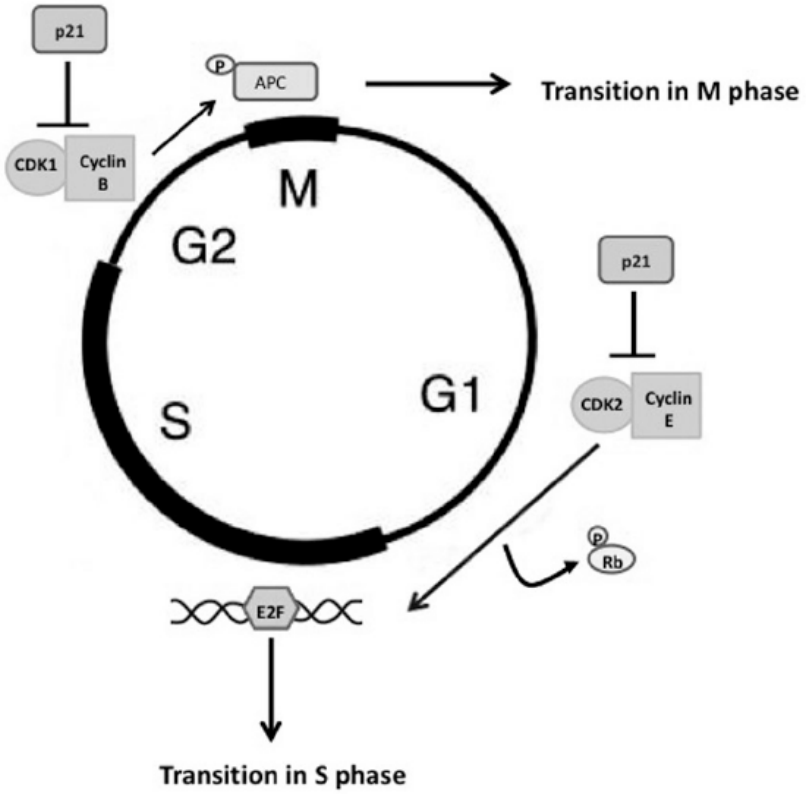

Figure 2: Inhibitory effects of p21 on the cell cycle at G1/S and G2/M checkpoints. p21 binds CDK2/CyclinE complex avoiding the phosphorylation of the retinoblastoma protein (Rb) and the consequent release of E2F, blocking the transition in S phase. p21 binds CDK1/cyclinB complex avoiding phosphorylation of substrates including APC (anaphase –promoting complex), responsible of the metaphase – anaphase transition.

the complex and interfering with CDK1 dephosphorylation on Thr14 and Tyr15 and with cyclin B phosphorylation on Thr161 [12]. CDK1/cyclinB complex promotes the transition in mitosis through the phosphorylation of some substrates as APC (Anaphase Promoting Complex), responsible of the transition from metaphase to anaphase. Finally p21 can act on G2/M checkpoint through the down-regulation of Emi1 (Early mitotic inhibitor). Emi1 regulates mitosis by inhibiting APC/C ubiquitin ligase complex (Anaphase Promoting Complex/Cyclosome) during S and G2 phases allowing a correct mitotic entry and preventing re-replication. It has been shown that Emi1 repression results in an APC/C-dependent degradation of cyclin A and B and G2 phase arrest [13].

Cell cycle progression can be influenced indirectly by p21 since it can interact with different transcription factors which are involved in cell cycle. Examples are Myc and p300/CBP (CREB Binding Protein). The proto-oncogene Myc is a transcription factor induced after mitogenic stimuli that allows the transition from quiescence to proliferation regulating genes linked to cell cycle. The interaction between p21 and Myc avoids the Myc association with other proteins involved in cell proliferation but at the same time Myc itself is able to block the binding between p21 and PCNA, thus inactivating p21 and promoting DNA replication [14]. p300/CBP coactivator proteins, two proteins involved in cell cycle and apoptosis regulation, are activated by p21 through the de-repression of a transcriptional repression domain (CRD1). p21 can also indirectly activate this complex by inhibiting CDK2/Cyclin E, a negative regulator of p300/CBP [15].

ANTI-APOPTOTIC ROLE OF p21

Inhibition of cell growth is the well-known role exerted by p21 even if several studies have reported different functions involved in apoptosis.

p21 can be considered a negative regulator of the p53-dependent apoptosis and it was suggested to be able to induce growth arrest if DNA damages are slight. When damages are more serious, p53 can directly induce apoptosis. In fact, prolonged treatments of cancer cells with DNA damaging agents, determine a p21 inactivation mediated by caspase-3 cleavage and reduction of p21 levels resulted in apoptosis [16]. The

same mechanism was evidenced after prolonged drug treatments in relation to drug dose. For example, Martinez *et al*., observed that doxorubicin in human prostate cancer cells induced p21 expression when administered at low doses, while p21 decreased with high doses treatment. Interestingly, in both conditions p53 expression resulted unchanged [17].

Depending on the presence or on the absence of p21, active p53 may promote growth arrest or apoptosis even if the exact mechanism of choice between one and the other has not yet been fully elucidated. The anti-apoptotic role of p21 is employed directly by determining cell cycle arrest or through the interaction with different pro-apoptotic proteins, by inhibiting their ability to induce apoptosis. Examples are procaspase 3, caspase 8 and caspase 10 and other factors that regulate apoptosis like the stress-activated protein kinase (SAPK) and the apoptosis signal-regulating kinase 1 (ASK1).

p21 also suppresses the expression of pro-apoptotic genes through Myc and E2F1 inhibiting their transactivation functions and it can even up-regulate genes coding protein with anti-apoptotic activity as galectin-3 and prosaposin [7].

Forster *et al*., described the anti-apoptotic role of p21 using a cell-based system expressing the BCR-ABL fusion protein that is crucial for pathogenesis of chronic myeloid leukaemia, in which p21 was deleted. The comparison of proliferation between wild type cells and p21 deleted cells revealed that, according to its classical role, p21 was able to reduce cell proliferation without influencing spontaneous apoptosis. On the contrary, the deleted cells showed an increased apoptosis after drug treatment. These results indicated a dual role of p21 since it was able to attenuate cell proliferation and drug-induced apoptosis [18].

A p21 antisense oligodeoxynucleotide treatment was used in breast cancer overexpressing p21 and characterized by a poor prognosis. The transfection determined a decrease of p21 expression with cell proliferation inhibition. According to the anti-apoptotic p21 function this reduction was associated to apoptotic increase in absence of drug treatment [19].

The anti-apoptotic effects of p21 have been tested also on human leukemic cells treated with a specific inhibitor of c-Jun N-terminal kinase (JNK) known for its anti-inflammatory and anti-cancer capacities (SP600125). This drug was able to increase p21 expression, preventing endoreduplication through inhibition of CDK2 kinase activity. SP600125 was also able to promote p21 phosphorylation *via* PI3K (PhosphatidylInositol 3-Kinase)/Akt pathway, a modification that prevents p21 binding to PCNA and determines the direct inactivation of caspase-3, an executor caspase of apoptotic process. Moreover, phosphorylated p21 can no longer inhibit cell-cycle progression inducing DNA synthesis [20].

Other studies demonstrated that the anti-apoptotic role of p21 is exerted by inhibiting initiator caspases as described in a study on DR4-induced apoptosis. DR4 belongs to TNF-related apoptosis-inducing ligand (TRAIL) receptor family and it is able to induce apoptosis through its cytoplasmic domain that determines caspase 8 and 10 cleavage. It has been shown that p21 overexpression can block the DR4-activated apoptosis before caspase cascade activation just inhibiting caspase cleavage [21].

PRO-APOPTOTIC ROLE OF p21

A large number of studies have shown that p21 in cancer plays a role in tumour suppression. p21 can promote apoptosis depending on particular cellular stresses when it results up-regulated and its expression is not related to the presence of a functional p53. The mechanisms involved in the p21-dependent apoptosis are not well understood and they seem to be related to the cell type and to the environment.

Several studies focused the pro-apoptotic role of p21 depending on specific systems or after drug treatments. The first evidences became from studies performed on human glioma cancer cell lines in which cisplatin treatment resulted in apoptosis increase related to p21 up-regulation in either a p53-dependent or independent manner with a p21 accumulation in the nucleus [22, 23]. Another study carried out on ovarian carcinoma reported that ectopic expression of p21 promoted cell growth inhibition with apoptosis increase. The effect was not related to p53 since it was observed also in a p53 defective cell line. Furthermore, ectopic expression of p21 enhanced the cytotoxic effect of the drug allowed decreasing the amount of cisplatin needed to achieve the same cytotoxic effects [23]. Both these studies indicated that p21 increased the susceptibility of cancer cells to cisplatin-induced apoptosis and suggested that it may be considered for the treatment of tumours lacking of functional p53.

A relationship between p21 expression and the cytotoxic effect of cisplatin has also been reported by

Baldi *et al*., who demonstrated an increase of p21 expression in mesothelioma cell lines with a functional p53, after a piroxicam/cisplatin-combined treatment. p21 over-expression determined an apoptotic increase that was mainly related to its nuclear accumulation evident in the combined treatment. Furthermore, apoptosis was tightly linked to the presence of p21 since silencing experiments impaired the functionality of the combined treatment [24]. This treatment was also shown to determine a tumour regression *in vivo* with survival increase, dependent on p21 up-regulation [25].

Different drugs can trigger apoptosis through p21 up-regulation. For example p21 expression is needed for apoptosis induced by oxysterol, an oxidized lipoprotein involved in atherosclerotic lesions. Analysis of mouse fibroblast cells lacking of p21 showed reduction in apoptosis in response to multiple stimuli, while p53 deficiency did not show to alter the apoptosis rates indicating a p53-independent mechanism [26]. Other studies in breast cancer cell lines, demonstrated that ectopic expression of p21 in MCF-7 and T47D, promoted a reduction of drug-resistant colonies, avoiding cell division or complete cytokinesis and allowing apoptosis [27]. Choi *et al*., showed that taxol treatment on MCF-7 and MDA-MB-231 promoted an accumulation of cells in G2/M and sub-G1 accompanied by inhibition of cyclin A and cyclin B1 and by significant induction of p21 [28].

Histone deacetylase inhibitors (HDAC) strongly activate the expression of p21 through the Sp1/Sp3 sites in p21 promoter competing for binding to p53. Trichostatin A was shown to induce apoptosis in human gastric and oral carcinoma cell lines by modulating the expression of cell cycle regulators and of proteins that regulate apoptosis. This molecule can activate the expression of p21, Bak and Bax, reducing the expression of p53 and E2F [29]. SAHA (Vorinostat), another HDAC inhibitor, is a potent inductor of p21 shown to be effective in several tumours through acetylation and methylation modifications in the p21 promoter, that lead to an increase of the region accessibility [30, 31]. More recently it was reported that L-Carnitine acts as an endogenous HDAC inhibitor being able to inhibit human hepatoma cell growth and cell death both *in vitro* and *in vivo* through p21 up-regulation. This molecule selectively induces p21 acetylating histone H3 in the promoter region. The activation is also related to a dramatic decrease of phosphorilated Rb protein, a consequence of the p21 cyclin inhibition [32].

Pro-apoptotic role of p21 has also been related to the TNF-α activity, a member of the TNF family of death receptors. It has been shown that TNF-α modulates apoptosis in limphocytes through the accumulation of p21 in a p53-dependent manner [33]. p21 activation was also reported in multipotent stromal cells preactivated with TNF-α and able to induce apoptosis in a xenograft model of human breast cancer [34].

Apoptosis induced by p21 can be realized in p53-dependent or independent manner and by a direct activation of pro-apoptotic proteins. A study performed on ovarian cancer cells p53-deficient has demonstrated that p21 expression is not needed for p53-mediated apoptosis and that its up-regulation can be independent from p53. In fact, adenovirus-mediated p53 or p21 expression showed apoptosis in both cases even if a higher increase was recorded after p53 induction. In addition, unlike p53, p21 induced apoptosis did not activate Bax and Bcl-2 suggesting the involvement of a distinct apoptotic pathway [35]. Similarly, another study reported that induced overexpression of p21 in cervical cancer cell lines lacking of the normal p53 resulted in both growth inhibition and apoptosis. In these experiments over-expression of Bcl-2 could not affect the p21 apoptotic induction [36].

In human hepatocellular cancer cells p53 deficient, the overexpression of C6-ceramide - a membrane sphingolipid shown to be biologically active - determined apoptosis and overexpression of p21. In this system p21 induces the pro-apoptotic protein Bax to levels sufficient to inhibit the apoptotic inhibitor Bcl-2 that represents its counterpart [37].

These studies underline the importance of p21 in regulation or induction of apoptosis, suggesting a specific role of p21 in response to cancer treatments.

REGULATION OF p21 EXPRESSION

Cells respond to damage stimuli increasing the protein levels of the tumour suppressor p53 that acts as transcriptional activator for several target genes among which p21 is the best known. In normal cells there are low levels of p53, while they generally increase after a genotoxic stress. p53 protein undergo to post-translational modifications that stabilize it, activate it and allow its nuclei accumulation. In stressed cells p53 induces the constitutive expression of p21 through the specific binding to two highly conserved response elements located in p21 promoter region [38].

This expression promotes a specific anti-apoptotic effect resulting in cell growth arrest. There is a direct correlation among DNA damages and p53-mediated p21 transactivation since no increase of p21 is evident in absence of p53. The importance of this mechanism has been supported by experiments showing the increase of p21 mRNA after DNA damage by whole-body γ-irradiation only in p53 wild-type tissues [39].

In addition to the well-known p53-dependent p21 activation, p21 can be activated also in a p53-independent manner by a variety of molecules that are induced by different signalling pathways. Sp1 (Specificity Protein 1) is one of the well-studied transcriptional regulator of p21 belonging to the Sp/KLF (Krüppel-Like Factor) family. It binds p21 promoter through specific binding sites in response to various agents.

A p21 activation p53-independent after phorbol esters and okadaic acid treatment of U937, a human leukemic cell line, has been described. These drugs are able to induce macrophages differentiation after the p21 transcription that is stimulated through the binding of transcription factor Sp1 in the promoter region. Deletions or mutations of these sites dramatically reduced transcription induction after drug treatments [40]. Perifosine, an anti-tumoural agent of the alkylphospholipids (ALKs), is another drug able to activate p21 transcription in a p53-independent manner. This drug activates the MAPK (Mitogen-Activated Protein Kinase) pathway that, inducing the phosphorylation of Sp1, promotes Sp1 binding to the p21 promoter. Again the specificity of the induction was demonstrated by the loss of p21 activation after sites-specific mutations [41].

The p21 induction in a p53-independent manner can be related to the binding of transcription factors at specific elements in p21 promoter. This binding can be enhanced by several anticancer drugs such as the histone deacetylase (HDAC) inhibitors.

Histone deacetylase (HDAC) inhibitors are a new class of anticancer agents able to modulate transcriptional activity and to induce cell cycle arrest and apoptosis in tumour cells. HDAC inhibitors specifically activate the expression of some genes as p21, by increasing histone acetylation around the promoter and at the Sp1 binding sites. So they inhibit cell cycle progression and promote cell death in tumours [42].

Other molecules are able to act as transcriptional p21 activators. The p300 and CBP (CREB-binding protein) coactivators have histone acetyl transferase activity and can transactivate p21 promoter in combination with other transcription factors as MyoD or BETA2, two members of the basic helix–loop– helix (bHLH) family protein.

MyoD is a myogenic-specific regulatory factor that induces p21 expression during skeletal muscle differentiation. This induction occurs in p53-independent manner and it is related to the terminal cell cycle arrest [43, 44]. It has been shown that p300 is an important factor whose activity is required to sinergize the transactivation of p21 mediated by MyoD [45]. MyoD also induces cell cycle arrest transactivating p21 and Rb during miofiber denervation, a process that can determine muscle atrophy. Activation of p21 during the early stages of denervation avoid apoptotic cell death so preventing muscle atrophy [46].

Activation of p21 is also involved in the terminal differentiation of the intestine epithelial cells. In this context p21 is transactivated by BETA2 and the concomitant expression of p300 enhances the BETA2-dependent p21 transcription. In this context induction of p21 coordinates terminal differentiation of these cells promoting their withdrawal from the cell cycle [47].

Other transcription factors such as AP-2, E2Fs and STATs can promote p21 transcription in response to different agents. AP-2 (Activator Protein-2) is a gene family of transcription factors involved in development, differentiation and transformation. It was shown that AP-2 overexpression in several cancer cell lines determined p21 activation and resulted in cell cycle arrest. AP-2 is able to activate p21 expression binding specific sites in the promoter region and mutagenesis of these sites resulted in inhibition of p21 activation mediated by AP2 [48].

E2F is a family of transcription factors that regulate genes involved in DNA replication and in cell cycle progression. Some members of this family such as E2F-1 induced endogenous p21 expression interacting with promoter region and this expression promoted cell cycle arrest in S phase by the inhibition of CDK2/cyclinE complex [49].

STAT proteins (Signal transducers and activators of transcription) are involved in cell growth suppression in response to citokine IFN-γ (interferon-gamma). They can mediate growth inhibition by up-regulating p21 by

recognizing and binding the cognate responsive elements located in the promoter [50].

Finally p21 can be activated by other molecules that act with mechanisms not well understood. Yang *et al*., have evidenced that anti-tumoural effects of JKA97, a novel small anticancer compound, is exerted in a p53-independent manner with unknown mechanism since this drug in human breast cancer cells induces apoptosis and inhibition of cell proliferation through the up-regulation of p21 expression, independently of the cellular p53 status [51]. A different p21 activation, p53-independent and not linked to transcriptional regulation, was mediated by Ccr4d, a protein involved in mRNA stability. Ccr4d inhibits cell proliferation and promotes cell cycle arrest at G1 phase, activating p21 expression. The regulation is exerted through the Ccr4d binding to p21 mRNA 3′-UTR that allows to the stabilization of p21 mRNA [52].

p21 EXPRESSION IN CANCER

Human cancers show different expression of p21 that depends on the cellular context and conditions, often with controversial results. Since p21 is one of the main targets of p53, it has been widely analysed in different human cancers in order to understand the existence of a correlation between its expression and prognosis.

The first study supporting the idea that p21 could act as a tumour suppressor derived from analyses of p21-null mice that were phenotypically normal but developed spontaneous tumours with age. This late cancer onset excluded the possibility that p21 could be regarded as the only responsible for tumourigenesis [6], indicating the importance of a cooperation among CKIs in cancerogenesis. In fact, p21 deficiency resulted more tumourigenic when associated to the loss of p27, causing spontaneous and aggressive cancerogenesis [53].

The absence of p21 promotes a major susceptibility to develop skin carcinoma and endocrine tumours. Many reports have observed that a loss or a down-regulation of p21 correlate with tumour progression and worse prognosis in different tumours as well as in small-cell lung [4], colorectal [5], and head and neck cancers [54]. In addition, in colorectal cancer p21 down-regulation has been associated to lymph node and liver metastasis that represent the main prognostic factors [55].

The analysis of the relationship between p53 and p21 expression in cancer displayed conflicting results. Some studies reported a negative correlation between the presence of mutated p53 and p21 expression [56, 57], while others did not find association between p21 expression and mutated p53 [58]. For these reasons the lack of p21 cannot always be related to p53 expression, nor p21 can ever be considered a prognostic factor. In contrast with these observations an increase of p21 expression was associated with carcinogenesis and negative prognosis in a variety of tumours, confirming the hypothesis of a possible role of p21 as oncogene. An overexpression of p21 was found in prostate [59], ovarian [60], cervical [61], breast [62] and esophageal carcinomas [63] and up-regulation of p21 has also been demonstrated in the majority of human gliomas where it is associated to drug resistance [64].

Unlike p53, that is often mutated in cancer, p21 mutations have been rarely reported especially considering large-scale cancer analysis. Various studies have showed that genetic variants in p21 can influence the risk to develop cancer. Two polymorphisms in p21 (C98A and C70T) have been linked to the increased risk of tumour recurrence in head and neck carcinoma [65]. Two other polymorphisms in the coding region of p21 (A168C and IVS2+16 C>G), associated to a polymorphism in the promoter region (4G/3G) have been related to increased susceptibility to esophageal cancer [66].

Despite the fact that somatic mutations were rarely found in human tumour, it is possible that genetic variants may influence p21 properties causing reduced protein expression or phenotypic variants.

SUBCELLULAR LOCALIZATION OF p21

Subcellular localization of p21 protein can influence its function (Figure **3**). In fact, p21 can be localized both in nucleus and cytoplasm and the different subcellular localizations have been addressed to associate them with cancer development. p21 localization can be influenced by post-translational modifications mainly determined by phosphorylations that might prevent p21 interactions with PCNA and CDK/cyclin complexes resulting in inhibition of cell cycle arrest.

In normal conditions p21, as other CKIs, is localized in nucleus. The nuclear localization is due to the NLS (Nuclear Localization Signal) sequence placed at C-terminal domain of the protein. This signal allows p21 translocation in nucleus through the NLS recognition by importins that determine the consequent passage

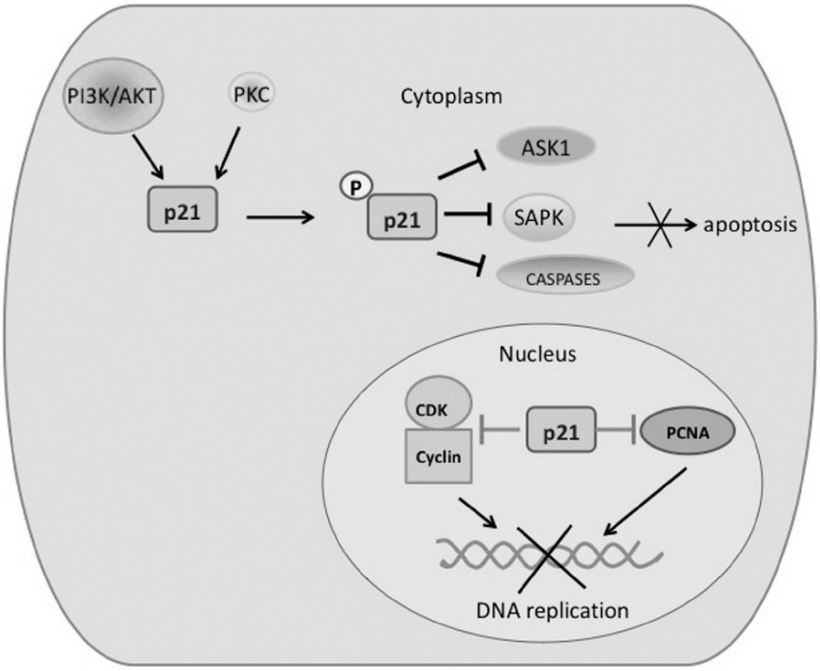

Figure 3: Different p21 functions in the subcellular compartments. In nucleus p21 inhibits CDK/cyclin complexes and PCNA avoiding cell cycle progression and DNA replication. In cytoplasm, post-translational changes of p21 by PI3K/Akt and PKC allow p21 to inhibit caspase cascade and interact with different pro-apoptotic proteins as SAPK and ASK1.

across nuclear pores [67]. In this cellular compartment p21 executes its physiological function of growth inhibitor, blocking the cells at G1 phase. In this compartment p21 also plays a role in regulation of the G2/M checkpoint, inhibiting CDK1 through a direct binding with CDK/cyclin complex.

In the nucleus p21 plays a role in senescence. Senescence is a cellular process induced after telomeres shortening (cellular senescence) or after premature DNA damage induced by several stress factors (stress-induced premature senescence). Senescent cells remain in a permanent cell cycle arrest without proliferating or undergoing to apoptosis.

p21 up-regulation is one of the first markers that can be detected at the initial stages of senescence and its protein levels increase proportionally to DNA damage. It has been reported that after ionizing radiation exposure of human fibroblasts, up-regulation of p21 determines a protection of cells against apoptosis and makes them more susceptible to undergo accelerated senescence [68]. Senescence is a multistep process in which p21 seems to be important mainly at the beginning of while the protein levels decrease over the time when p16, another CKI, acts on the terminal stages of growth arrest [69]. Similar results performed in cardiomyocytes in which senescence was induced by hypoxia resulted in a sustained increase of p21 expression as cells enter senescence. Subsequently, it was observed an up-regulation of p16 concomitant to p21 decrease. These experiments also showed that p21 is needed to trigger cellular senescence since the process cannot be activated in p21 silenced cells [70].

Although NLS mediates the acute regulation of p21 intracellular distribution, nuclear translocation can be inhibited by phosphorylation of Ser and Thr residues near the NLS sequence. This modification can be mediated by different kinases such as Akt or PKC that increase p21 levels in cytoplasm and promote p21 function as a positive modulator of cell survival.

Akt pathway is implicated in many late stage of tumours, it is activated by a variety of proteins as PI3K (Phosphatidylinositol-3-kinase) and modulates the activity of several transcription factors including p21. Akt can phosphorylate p21 at Thr145 in NLS and in PCNA binding site, causing citoplasmic accumulation and inhibiting the interaction with PCNA and CDK/cyclin complexes.

Similarly, Ser153 phosphorylation mediated by PKC destabilizes p21 altering the binding of interacting proteins at the C-terminal region and favoring its cytoplasmic localization.

Subcellular localization of p21, can be influenced by different factors. BCCIP, a protein interacting with p21,

contributes to determine its subcellular localization. BCCIP binds the C-terminal domain of p21, promoting accumulation of p21 in nucleus and its inhibitory activity towards CDK2/cyclin complex. Moreover BCCIP being required for p53 transcriptional activity can regulate the expression of p21 [71].

Ultimately, p21 in the nucleus is mainly related to the cellular stress response when p53 acts as p21 transcriptional activator. Also some drug treatments can influence p21 nuclei accumulation. For example, it has been reported that in mesothelioma cell lines the combined treatment piroxicam/cisplatin determined an apoptosis induction related to an increase of the p21 nuclear localization. This result supports the hypothesis that p21 in the nucleus has a dual role being anti-proliferative and pro-apoptotic [24].

Cytoplasmic accumulation of p21 is known to be related to the anti-apoptotic role and a positive relationship has been found between this subcellular localization and tumour development, suggesting that cytoplasmic p21 can promote tumour growth.

The importance of p21 cytoplasmic localization on the proliferation has been shown transfecting hepatic carcinoma cells with a p21 construct mutated in NLS sequence. Cells expressing mutated NLS showed a cell proliferation increase and a major anti-apoptotic capacity probably related to the specific phosphorylation of residues. This modification is subsequent to the cytoplasmic translocation and it is needed to allow the association of p21with anti-apoptotic proteins [72].

The association between cytoplasmic localization and apoptotic inhibition was reported by Xia *et al*., who used two cell lines OV2008 and C13 characterized by a selective expression of p21 in the nucleus or in the cytoplasm respectively. Cisplatin treatment of the cells induced a p21 cytoplasmic translocation only in the OV2008, where there was also an apoptotic induction. Cisplatin treatment induced apoptosis in C13 only after inhibition of the p21 translocation in cytoplasm [73].

Subcellular localization of p21 can be altered modulating NUPR1, a protein up-regulated in response to cellular stress that plays a role in tumour progression. The relationship between NUPR1 and p21 localization was analysed in two engineered stable breast cancer cell lines, silenced for NUPR1 (MCF10AT) or that constitutively express it (SUM159). Only SUM159 cells showed an up-regulation of p21 and a cytoplasmic localization that was related to a drug resistance [74].

p21 function can be modulated not only at transcriptional but also at post-translational level. In particular phosphorylations seem to play a fundamental role in protein functions, stability and subcellular localization.

The most studied phosphorylations are related to residues of Threonine 145 (Thr145), Serine 153 (Ser153) and Serine 146 (Ser146).

Thr145 can be phosphorylated by serine/threonine kinase Akt and in this conformation p21 loses the capacity to bind with PCNA shifting from nucleus to cytoplasm [75]. This condition also promotes an increased degradation of p21. Xia *et al*., have demonstrated that Thr145 phosphorylation is related to worse overall survival in HER2/neu-overexpressing breast cancer cells, evidencing that in this form p21 is prevalently localized in the cytoplasm. The re-localization of p21 is probably due to the proximity between Thr145 and p21 NLS since the phosphorylation prevents the interaction of NLS and importins that is requested for the nuclear import [76].

Ser153 is another important residue that can be phosphorylated by PKC (Protein Kinase C). Earlier reports have observed that phosphorylation of Ser153 prevents p21 nuclear accumulation promoting translocation in cytoplasm, as demonstrated using the calmodulin, a molecule that prevents this modification and stabilizes the protein in nucleus [77, 78].

Phosphorylation at sites Ser146 [79], Ser160 [80] or Ser130 [81] do not allow the cell cycle progression since the binding to PCNA or to CDK2 is inhibited but the translocation into the cytoplasm is not promoted. In particular Ser146 can be phosphorylated by Akt1 or protein kinase C and this modification also determines p21 stabilization increasing cell survival [82].

TARGET p21 AS A CANCER THERAPY

Cancer studies have indicated that p21 expression combined with p53 status can be used to predict the clinical outcome. Many reports suggested that p21 overexpression in cancer was associated to a better prognosis. For example, studies performed in ovarian and uterine cancers reported an inverse correlation between p21 expression and cell proliferation and a strong association of low p21 expression with high grade of the tumour and poor survival [83, 84].

Hafkamp *et al*. analyzed the expression of cell cycle proteins in tonsillar carcinoma induced by Human papillomavirus. They showed a p21 overexpression associated to a down-regulation of pRb and cyclin D1 independent from p53 expression. Furthermore, p21 overexpression resulted positively associated to a more favorable prognosis [85]. In this regard, the use of molecules able to activate p21 could be helpful to develop new anticancer therapies. Examples are the HDAC inhibitors that enhancing p21 activity can trigger apoptosis in cancer cells. The induction is achieved binding the Sp1/Sp3 binding sites in the p21 promoter competing for p53 binding [42].

Similar effects can be obtained acting indirectly on p21 by targeting molecules that mediate p21 apoptotic functions. For example, E2F gene transfer combined with drug treatments has been shown to sensitize cancer cells to apoptosis in several tumours [86, 87].

Recent studies have reported an anticancer effect due to a p21 induction achieved by synthetic dsRNAs that target promoter regions - a phenomenon known as RNA-induced gene activation. This strategy has been demonstrated to be successful in bladder cancer cells in which a specific dsRNA determines p21 up-regulation with a concomitant Bcl inactivation and activation of caspase-3 and caspase-9 [88].

Another approach is based on the senescence induced by p21, since this process also determines tumour regression. Several studies achieved tumour senescence inactivating the oncogene Myc by generating transgenic mice that conditionally overexpress Myc. The presence of Myc was reported necessary for the initiation and maintenance of tumorigenesis in transgenic tumour models of lung adenocarcinoma and lymphoma. In these models induction of Myc in the lung epithelium by oral administration of doxycycline resulted in tumorigenesis while Myc inactivation resulted in tumour regression and it was associated to a decrease in Akt phosphorylation [89]. Myc suppression has been reported involved in tumour regression in lymphoma, osteosarcoma, and hepatocellular carcinoma. The senescence was associated to up-regulation of several cell cycle inhibitors including p21 [90]. Myc down-regulation was also associated to the treatment with ascofuranone, an antibiotic that inhibits cell proliferation. It has been shown that the antitumour activity of this molecule was related to the ability of inducing p21 expression and G1 arrest through p53-independent suppression of Myc expression [91].

Finally, a different therapeutic approach to achieve tumour regression could be related to p53 repair. It is known that loss of p53 function may play a role in tumour development and mainteinance and that deletion or mutation of p53 is a common feature in human cancers. A mouse model of liver carcinoma was used to demonstrate that a functional p53 restoring determined cell cycle arrest *in vitro* and a complete tumour regression that was related to senescence induction [92]. Similarly, using a mouse model with an inducible p53 allele it was shown that only mice lacking of p53 developed tumours, while p53 restoration allowed tumour regression *in vivo*. p53 reactivation resulted in apoptotic increase and related to caspase-3 and p21 induction [93].

All these studies indicate that the understanding of the molecular mechanisms that in tumours correlate p21 up-regulation with apoptosis or growth inhibition will be important to develop novel therapeutic strategies.

CONCLUSIONS

The reported results underline the dual role of p21 since it can have tumorigenic or anti-tumorigenic activity.

It is now clear that the different role is related to its subcellular localization that is also influenced by post-translational modifications. In the nucleus p21 has a tumour suppressor activity because it negatively regulates the cell cycle progression acting on CDK/cyclin complexes suppressing their activities. Other studies have also shown a direct pro-apoptotic role of p21 exerted mainly in the nucleus, but the exact mechanisms have not deeply investigated yet. On the contrary, p21 in the cytoplasm facilitates cell proliferation and inhibits apoptosis acting as oncogene.

Studies on p21 expression in cancer remain controversial. In fact, increased expression of p21 was found in different human cancers where often it was correlated to cancer invasiveness and aggressivity. On the other hand, reduced levels of p21 were related to increased risk of tumour development. Moreover, many studies reported that p21 can be induced by different anti-cancer drugs allowing an increase of citotoxicity.

In future, a better knowledge on p21 in cancer will be needed to understand the mechanisms regulating its expression and biological effects. In the light of next investigations in different cellular conditions and

contexts, p21 could be examined as a prognostic factor or as a potential therapeutic target in tumour treatments.

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