

Review Article

Tumor Suppressors and Cell-Cycle Proteins in Lung Cancer

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The cell cycle is the cascade of events that allows a growing cell to duplicate all its components and split into two daughter cells. Cell cycle progression is mediated by the activation of a highly conserved family of protein kinases, the cyclin-dependent kinases (CDKs). CDKs are also regulated by related proteins called cdk inhibitors grouped into two families: the INK4 inhibitors (p16, p15, p19, and p21) and the Cip/Kip inhibitors (p21, p27, and p53). Several studies report the importance of cell-cycle proteins in the pathogenesis and the prognosis of lung cancer. This paper will review the most recent data from the literature about the regulation of cell cycle. Finally, based essentially on the data generated in our laboratory, the expression, the diagnostic, and prognostic significance of cell-cycle molecules in lung cancer will be examined.

1. Introduction

Lung cancer remains a major health challenge in the world. Despite improvements in staging and the integrated application of surgery, radiotherapy, and chemotherapy, the 5-year survival rate for individuals with lung cancer is only about 15% [1]. Histologically, 80% of the lung cancers are diagnosed as non-small-cell lung cancer (NSCLC), whereas the remaining 20% of cases are diagnosed as small-cell lung cancer (SCLC). On the basis of cell morphology, adenocarcinoma and squamous cell carcinoma are the most common types of NSCLC. The current staging system for NSCLC is based upon the size and location of the primary tumor (T), the involvement of regional lymph nodes (N), and the presence of distant metastases (M) [1]. The standard treatment of patients with stage I NSCLC (T1-2, N0, M0) is resection of the primary tumor alone (no adjuvant therapy) [2]. Survival for patients with stage I disease ranges between 40 and 70%, and the failure is due to distant recurrences [3]. This suggests that a significant proportion of patients with stage I NSCLC may actually be understaged. Therefore, if correctly identified, these patients may benefit

from adjuvant therapy in addition to resection, with a predictable improvement in the survival rates. Indeed, to identify patients with stage I NSCLC who might benefit from adjuvant therapy, investigators have attempted to identify factors predicting poor prognosis. These studies included analysis of performance status, histologic subtype, size of the primary tumor, the degree of tumor differentiation, mitotic rate, and evidence of lymphatic or vascular invasion [4–8]. However, all of these factors have failed, to date, to precisely identify a group of stage I patients who would benefit from adjuvant therapy. Cigarette smoking remains the main risk factor for lung cancer, accounting for about 90% of the cases in men and 70% of the cases in women [9].

Our research group has investigated in the last years the possible involvement of several molecular mechanisms, such as cell cycle and apoptosis regulators, oncogenes and tumor suppressor genes, cell adhesion molecules, in the pathogenesis and progression of lung cancer [10–20]. Goal of this paper is to summarize some of the most recent findings about the regulation of cell cycle and about the role of cell-cycle proteins in lung-cancer pathogenesis and progression.

2. Cell Cycle

The cell cycle is the cascade of events that allows a growing cell to duplicate all its components and split into two daughter cells; it consists of four distinct phases: G1 phase, S phase, G2 phase (collectively known as interphase), and M phase. High fidelity duplication of DNA in each cell during the S phase and the proper migration of the duplicated chromosomes in mitosis are highly regulated processes.

Cyclins and cyclin-dependent kinases (CDKs) are the two critical classes of molecules involved in the regulation of cell-cycle progression. These proteins form a heterodimer in which cyclins are the regulatory subunits and CDKs are the catalytic subunits; when the complex is activated from external signals, CDKs activate or inactivate downstream target proteins to orchestrate coordinated entry into the next phase of the cell cycle [21].

In normal cells, CDKs are expressed throughout all the cycle; however, each cyclin protein has a restricted period of expression, and this limited expression of each cyclin protein is due to cell-cycle-dependent regulation of both cyclin gene transcription and protein degradation.

The cyclin/CDK complex is regulated by a number of phosphorylation and dephosphorylation events, resulting either in activation or inhibition of kinase activity [22]: phosphorylation is carried out by *cyclin-activating kinase (CAK)*, and dephosphorylation is mediated by members of the *Cell Division Cycle 25 family (Cdc25)* of dual-specificity protein phosphatases. The mammalian Cdc25 family consists of three members: Cdc25A, Cdc25B, and Cdc25C. Cdc25A promotes entry into S phase by acting on cyclin A/CDK2 and cyclin E/CDK2 and is required for DNA replication [23–26]. Cdc25B activation occurs during S phase and peaks during the G2 phase [27, 28]. Both Cdc25B and Cdc25C play roles in the G2/M transition. Cdc25C dephosphorylates cyclin B1/cdc2 and is essential for progression through the G2/M phase of the cell cycle [29].

Decision to enter S phase is made in mid-to-late G1 and is called the *restriction point*. Molecular switch from G1 to S phase and targets of the G1 phase cyclin/CDK complexes are members of the retinoblastoma protein (RB) family: RB, p107, and p130 [30]. RB can function as either a transcriptional repressor or a transcriptional activator depending on its phosphorylation state and the proteins with which it binds: when hypophosphorylated and bound to the E2F family of transcription factors, it works as a transcriptional repressor [31]. The E2F family mediates transcription of genes required for DNA synthesis, including cyclin E, cyclin A, cyclin B, dihydrofolate reductase, and thymidine kinase [32]. Sequential phosphorylation of RB by cyclin D/CDK4/6 and cyclin E/CDK2 complexes inhibits the repressor activity of RB, as it results in the dissociation of E2F and RB, and S phase entry. As cells progress into S phase, maintenance of RB hyperphosphorylation is necessary for the successful completion of DNA replication [33]. Mutations that affect the RB signaling pathway have been identified in the majority of human cancers [34].

CDKs are also regulated by a group of functionally related proteins called *CDK inhibitors*. The CDK inhibitors

are grouped into two families: the INK4 inhibitors (p16, p15, p19, and p18) and the Cip/Kip inhibitors (p21, p27, and p53). The INK4 family specifically inhibits CDK4 and CDK6 activity during the G1 phase of the cell cycle, while the Cip/Kip family inhibits CDK activity during all phases of the cell cycle. Both families of these inhibitors can arrest cells in the G1 phase of the cell cycle by inhibiting the activities of CDKs and preventing their ability to phosphorylate and inactivate RB and other RB-family proteins [21].

The *S phase* of the cell cycle is the stage in which occurs the DNA replication. At the beginning of the S phase, each chromosome is composed of one coiled DNA double helix molecule; at the end of this stage, each chromosome has two identical DNA double helix molecules and therefore is composed of two sister chromatids. During this phase, the centrosome is also duplicated [35, 36]. It is proposed that replication origins are in two different states during the cell cycle: one during G1 phase, before DNA replication begins, when a multiprotein prereplicative complex (*pre-RC*) assembles on the origin. The second state exists from the initiation of S phase to the end of M phase, when a postreplicative complex (*post-RC*) is present at the replication origins.

Initiator proteins required for pre-RC formation include the origin recognition complex (ORC), MCM proteins (that forms the replicative helicase), Cdc6, and Cdt1 (required to load MCM proteins on chromatin) [37–40]. This multiprotein complex is activated at the G1/S transition; subsequently, DNA replicating proteins such as DNA polymerase α and β are recruited to initiation sites [27]. An increase in CDK2 and hcdc7 activity at the G1/S transition triggers initiation and converts the origin to the post-RC state. The CDK cycle controls the two states at replication origins, couples the initiation of S phase to the completion of M phase, and prevents rereplication events from occurring during a single round of the cell cycle [41, 42].

As cells enter into *G2 phase*, the cyclin B/cdc2 complexes are kept inactive by phosphorylation. At the end of this phase, the cells are ready to enter into mitosis, and cyclin B/cdc2 complexes are activated by dephosphorylation [43]. The enzyme that dephosphorylates and activates cdc2 at the end of G2 and initiates mitosis is Cdc25C [44]. This transition point is one of the most important during the progression of cell cycle, and different mechanisms control this stage. Cdc25C is localized in the cytoplasm during interphase and enters the nucleus just before mitosis. Also cyclin B1 is in the cytoplasm during S phase and G2 phase and is translocated to the nucleus at the beginning of mitosis [45]. It is thought that the precise regulation of cyclin B1 localization prevents premature mitosis during interphase, while allowing regulated access of cyclin B1/cdc2 complexes to their nuclear substrates at the onset of mitosis. Although less well understood, Cdc25B and A/Cdc2 also play a role at the mitotic transition.

As cells enter *mitosis*, phosphorylation of key components causes significant changes in the architecture of the cell, and this phosphorylation is due mainly to cyclin B/cdc2 activity [46]. This complex induces changes in the microtubule network, in the actin microfilaments, and in the nuclear lamina [47]. Other cyclin B/cdc2 substrates include

histone H1 and microtubule-associated proteins such as MAP4, MAP2, and stathmin [48]. The family of polo-like protein kinases (Plks) also plays a critical role in several mitotic events [49]. They are critical for the formation of a bipolar spindle. It is proposed that Plks initiate the onset of mitosis by activating Cdc25C. Plks are also important regulators of mitotic exit.

Mitotic exit requires sister chromatid separation, spindle disassembly, and cytokinesis. The initiation and coordination of these processes are controlled by degradation of key regulatory proteins. The mediator of this protein destruction is a multisubunit protein called the *anaphase-promoting complex (APC)* or cyclosome [50]. Key APC substrates are the mitotic A- and B-type cyclins. Cyclin A is degraded in metaphase, whereas B-type cyclins are degraded when cells enter anaphase [51]. Cyclin B1 destruction starts as soon as the last chromosomes are aligned on the metaphase plate and is complete by the end of metaphase [52]. Another group of APC substrates are proteins that function as anaphase inhibitors. During G2, sister chromatids are held together by proteins called cohesins, which require inactivation by APC for anaphase initiation [53]. Overall, the APC regulates two different steps in mitosis. First, sister chromatid separation is triggered by destruction of the anaphase inhibitors, after which spindle disassembly and mitotic exit are initiated by the degradation of mitotic cyclins. These two steps allow the cell to couple the exit from mitosis to the prior completion of anaphase.

3. Cell-Cycle Checkpoint

Cell-cycle checkpoints are signal transduction pathways monitoring the successful completion of events in one phase of the cell cycle before proceeding to the next phase. Cell-cycle checkpoints contain sensor proteins that scan chromatin for partially replicated DNA, DNA breaks, or other abnormalities. Sensor proteins are thought to translate DNA-derived stimuli into biochemical signals that modulate specific downstream target proteins and activate signaling pathways involved in DNA repair and cell-cycle arrest [54]. Further, when cellular damage is irreparable, checkpoint signaling could eliminate potentially hazardous cells by permanent cell-cycle arrest or apoptosis.

The physiological relevance of these signaling pathways is supported by their evolutionary conservation and the finding that the major consequence of their alteration in humans is tumorigenesis [54]; in fact, the loss of cell-cycle checkpoints is a universal alteration identified in human cancer. Although numerous genetic alterations can result in loss of normal checkpoints, the hope is that common strategies will be developed against a wide variety of cancers.

3.1. G1/S Phase Checkpoint. The G1 cell-cycle checkpoint prevents damaged DNA from being replicated and is the best understood checkpoint in mammalian cells. Progression of cells through early G1, across the restriction point into late G1 and then into S phase, requires the coordinated regulation

of multiple positive and negative factors [55]. Cyclin D-CDK4/6 complexes promote early G1 progression, but cyclin E (or cyclin A)-CDK2 (or CDK1) activity is required to inactivate RB by hyperphosphorylation to transit the restriction point into late G1 phase [56]. RB inactivation results in release of E2F transcription factors and induction of late-G1-specific genes, including dihydrofolate reductase (DHFR), Emi1, and cyclin A [57]. Cyclin A-associated kinase activity is required to initiate DNA synthesis, prevent rereplication, and enter mitosis.

Although cyclin A is transcriptionally induced by E2Fs at the restriction point, cyclin A protein does not accumulate until the late G1/S phase transition due to ubiquitination by the anaphase promoting complex (APC) and subsequent proteolysis by the 26S proteasome. APC is active throughout G1 phase by association with Cdh1 (APCCdh1), an activator that confers substrate specificity [58]. Prior to initiation of S phase, APCCdh1 is inactivated by the binding of Emi1 to Cdh1, resulting in stabilization of cyclin A, activation of cyclin A-associated kinase activity, and subsequent inactivation of Cdh1 by phosphorylation [59]. Thus, tight regulation of cyclin E- and A-associated kinase activity results in a coordinated G1 cell-cycle progression [60].

If DNA is damaged, the G1/S checkpoints prevent transition of cells into S phase. Due to its essential and rate-limiting role in G1/S transition, cyclin E/CDK2 is a key target for the DNA damage checkpoint [21]. Damage to DNA inhibits the action of CDK2 thus stopping the progression of the cell cycle until the damage can be repaired. If the damage is so severe that it cannot be repaired, the cell self-destructs by apoptosis. It is proposed that after exposure of cells to UV or IR, the level of Cdc25A phosphatase rapidly decreases. Cdc25A removes the inhibitory phosphorylation on CDK2 that is required for G1/S transition. After IR or UV exposure, Cdc25A is rapidly phosphorylated by Chk2 and Chk1, respectively. Chk-mediated phosphorylation triggers accelerated turnover of Cdc25A and thus inhibition of CDK2 [61]. An endpoint of this checkpoint signaling is inhibition of CDK2-dependent loading of Cdc45 onto the DNA prereplication complexes and thus inhibition of S phase [62].

Another important target for checkpoint signaling in cells that are in G1/S transition before the restriction point is the p53 tumor suppressor protein [63]. In normal, non stressed cells, p53 protein has short half-life as result of the rapid MDM2-mediated degradation of the protein after synthesis [64, 65].

After exposure of cells to stress, p53 phosphorylation changes and protein levels increase significantly. Transducers that are required for p53-mediated maintenance of G1 checkpoint arrest are the same as those required for activation of the checkpoint, namely, the ATM/ATR and Chk2/Chk1 kinases. Among the genes regulated by p53, the CDK-inhibitor p21WAF1/Cip1 plays a central role in G1 checkpoint by inhibiting CDKs that are essential for entry into S phase [66–68]. Thus, although ATM/ATR-mediated signaling can phosphorylate key targets Cdc25A and p53 within minutes after DNA damage, the impact of the signaling pathways regulated by Cdc25A and p53 on CDK2

activity and G1/S blockage are separated in time, due to the dependence of p53 signaling on transcription and protein synthesis.

Human cells also have evolved additional mechanisms to prolong a G1 cell-cycle checkpoint arrest. For example, after exposure of keratinocytes and melanocytes to physiological doses of UV radiation, there is an increase of the CDK-inhibitor p16INK4a [69]. This factor acts as tumor suppressor which inhibits the activities of cyclin D-dependent kinases, CDK4 and CDK6, and regulates the activities of RB [70]. Given the direct role that CDK-inhibitors play in regulation of the G1/S transition, it is not surprising that CDK-inhibitor function is often compromised in human tumors. The p16INK4A gene is the frequent target of mutations that ablate its function, including point mutations, promoter methylation, or homozygous deletions [71]. Likewise, many human breast cancers have reduced p27Kip1 protein expression or aberrant subcellular localization of the protein that has been correlated with more aggressive tumors [72, 73].

3.2. S Phase Checkpoint. The S phase checkpoint monitors cell-cycle progression and decreases the rate of DNA synthesis following DNA damage. The responses of cells that are already in S phase at the time of the DNA damage will be critical for optimal outcome of the cell. These cells must respond virtually instantaneously to halt initiation of new replication forks throughout S phase, and the first action to prevent wrong DNA synthesis is the activation of the ATM (ataxia telangiectasia mutated) and/or ATR (ATM and Rad3-related) protein kinase. Both of these proteins belong to a structurally unique family of serine-threonine kinases, but they generally respond to distinct types of DNA damage.

ATM is the primary mediator of the response to DNA double strand breaks (DSBs) that can arise by exposure to ionizing radiation (IR) [74]. For responses to other types of DNA damage, such as base damage caused by exposure to ultraviolet light or alkylating agents, the ATR kinase appears to be important for initiating the relevant signal transduction pathways [75].

Once ATM or ATR has been activated by the introduction of DNA damage, these protein kinases begin to phosphorylate substrates to help the cell arrest cell-cycle progression or repair DNA. As discussed previously, the phosphorylation of p53, MDM2, and Chk2 by ATM following DNA damage contributes to the arrest of cells in G1 before the restriction point. Among the proteins phosphorylated by ATM that contribute to arrest of cells in S phase are Nbs1, Brca1, SMC1, and FANCD2 [76–79]. The importance of this process in cancer formation in humans is suggested by the fact that many of these genes are mutated in familial cancer syndromes. For example, the cancer susceptibility syndromes Ataxia-telangiectasia, Nijmegen breakage syndrome, Fanconi's anemia, and familial breast/ovarian carcinoma syndrome are caused by inherited mutations in ATM, Nbs1, FANCD2, and Brca1, respectively.

3.3. G2 Checkpoint. In addition to activation of the G1/S and S phase checkpoints, DNA damage also activates checkpoint

arrest in G2 to prevent the passage of DNA lesions to two daughter cells during mitosis. At this stage, entry into mitosis is controlled by the activity of the cyclin-dependent kinase Cdc2. Maintenance of the inhibitory phosphorylations on Cdc2 is essential for G2 checkpoint activation. ATM and ATR indirectly modulate the phosphorylation status of these sites in response to DNA damage. These DNA damage checkpoint pathways all share common upstream signaling pathways made up of the ATM/ATR transducer and Chk2/Chk1 effector kinases [80].

Activation of the G2 checkpoint after genotoxic stress involves ATM-mediated phosphorylation and activation of the Chk1 and Chk2 kinases [81, 82]. It is proposed that direct inhibition of Cdc25 activity by Chk1 is sufficient for proficient checkpoint regulation of Cdc25 and that Cdc25C might be inhibited by another upstream kinase, Plk1 [83]. The activity of Plk1 is inhibited in the G2 phase of human tumor cells exposed to ionizing radiation, camptothecin, and doxorubicin. Further, expression of a mutant Plk1 in which residues necessary for Plk1 activation are altered, prevents Plk1 inactivation, and leads to G2 override in cells treated with doxorubicin [84].

In addition to a role in G1/S checkpoint function, p53-mediated signaling plays an integral role in maintenance of the G2 checkpoint delay after activation of the checkpoint. p53 is believed to exert G2 checkpoint responses through transcriptional upregulation of the downstream target genes p21, 14-3-3, and GADD45. Similar to its regulation of the cyclin D1/cdk4,6 or cyclin E/cdk2 complexes at the G1/S checkpoint, p21 can bind to and inhibit the cyclin B1/cdc2 complex and inhibit cyclin-activated kinase-mediated cdc2 activation [85]. The p53-dependent increase in 14-3-3 modulates the subcellular localization of the cyclin B1/Cdc2 complex, as the binding of 14-3-3 to cdc2 results in retention of the kinase in the cytoplasm [86]. The p53-mediated GADD45-dependent G2 arrest is induced only after specific types of DNA damage, as lymphocytes from GADD45 knockout mice failed to arrest after exposure to UV radiation but retained the G2 checkpoint initiated by ionizing radiation [87].

3.4. Spindle Checkpoint. The mitotic spindle checkpoint monitors spindle microtubule structure, chromosome alignment on the spindle, and chromosome attachment to kinetochores during mitosis [88]. The spindle checkpoint delays the onset of chromosome segregation during anaphase until any defects in the mitotic spindle are corrected. Unattached kinetochores are thought to be the source of the checkpoint signal, and mechanical tension at the kinetochore dictates whether the checkpoint is initiated or not [89]. Activation of the spindle checkpoint prevents mitotic progression through inhibition of the anaphase-promoting complex activator, Cdc20 [89]. Mediators of the spindle checkpoint pathway include the Mad2, Bub1, and Bub3 proteins [90]. Mad2 localizes to the kinetochores during prometaphase until alignment of the chromosomes occurs in metaphase and regulates mitotic exit by interaction with components of the APC machinery (such as Cdc20) that mediate anaphase

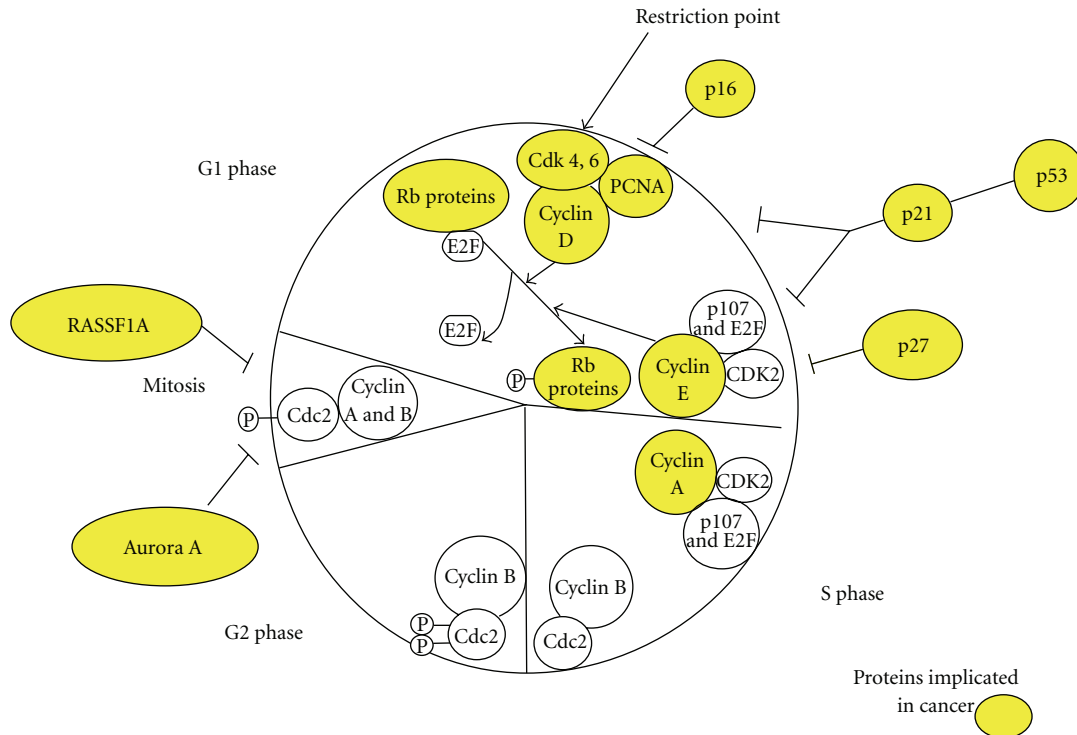


FIGURE 1: The cell-cycle clock. The most important proteins involved in cell-cycle regulation are depicted.

entry. Bub1 and Bub3 also localize to kinetochores and regulate chromosome/kinetochore interactions, and both are required for cell-cycle arrest after disruption of microtubule dynamics during mitosis. Inactivating mutations in Bub1 have been identified in human colon carcinoma cell lines, suggesting that disruption of the spindle checkpoint could occur during tumor progression.

Integral to cell-cycle regulation is the proper coordination of mitotic exit and subsequent S phase entry. After DNA synthesis, cells have a tetraploid (4N) DNA content that is reduced to a diploid (2N) DNA content in each daughter cell after successful completion of mitosis. Intact checkpoint pathways are needed to prevent the S phase entry of cells that have failed to properly segregate their chromosomes during mitosis. Cells with defective spindle checkpoint function can exit from mitosis with a 4N DNA content. These cells can inappropriately continue to the next cell-cycle division and, in the absence of a functional G1/S checkpoint, enter S phase with a 4N DNA content; this process is known as endoreduplication. Endoreduplication results in the generation of polyploid cells, that is, cells with a 4N or greater DNA content after mitotic exit. Cells that are RB-, p53-, p21-, or p16-deficient can endoreduplicate after microtubule inhibitor treatment [91, 92].

The G1 cell-cycle regulators, however, do not directly regulate the mitotic arrest induced by microtubule inhibitors; rather, absence of these proteins allows deregulated CDK2 activity, the precise control of which is required for normal cells to maintain proper coupling of mitotic exit and S phase entry [92, 93]. Thus, in addition to playing a role

in checkpoint function after DNA damage, proteins that mediate the G1/S checkpoint through regulation of cdk2 activity also prevent inappropriate S phase entry after an abnormal mitotic exit and are critical to proper coordination of S phase and mitosis.

In Figure 1, the most important cell-cycle molecules involved in cancer pathogenesis and progression are depicted.

4. Cell-Cycle Proteins and Lung Cancer

4.1. Components of the G1 to S Phase Transition in Lung Cancers. Concerning lung cancer, most of the studies about the cell-cycle regulation in this neoplasm have been performed on the G1/S phase. The retinoblastoma gene family consists of three members, the product of the retinoblastoma gene (pRb), which is one of the most studied tumor suppressor genes, and two related proteins, p130 and p107, which have been shown to be structurally and functionally similar to pRb [94]. Sequence analysis of these two proteins shows they share large regions of homology with pRb, especially in two discontinuous domains which make up the "pocket region" [95, 96]. The pocket domain is required for binding the three members of the Rb-family with several viral transforming oncoproteins, as well as with members of the E2F family [97]. Both p130 and p107, like pRb, display growth suppressive properties, although the growth arrests mediated by the three pocket proteins are not identical. This suggests that, although the different members of the retinoblastoma gene family may complement each other, they are not fully redundant

functionally [98, 99]. The Rb pocket proteins (pRb, p107, and p130) play a critical role in G1/S progression, at least in part, through binding and inactivation of factors (e.g., E2F) that promote transcription of genes required for DNA replication. Although p130, p107, similarly to pRb, interact with members of the E2F transcription family and have similar functional consequences, each pocket protein has a different temporal profile of interaction with different E2F/DP1 complexes. The binding of p130 to these complexes is detected predominantly during G0, 12–15 while that of p107 is detected during the G1 and S phases [98–104]. Thus, it is possible to propose a simple model in which the three members of the retinoblastoma gene family bind and modulate the activity of the E2F/DP complexes, as well as other transcription factors. In this model, the binding is regulated by different upstream signals such as cyclin/cdk complexes or viral oncoproteins. The flexibility of this pathway could explain the distinct activities of the three pocket proteins in the regulation of cellular division and cellular differentiation. Active (underphosphorylated) pRb can be inactivated and induced to release transcription factors when it is hyperphosphorylated (in mid-late G1) by cyclin/cdk4,6 complexes. In turn, the cyclin/cdk complexes are negatively regulated by two sets of inhibitors, the p21 family (p21^{Cip1/Waf1}, p27^{Kip1}, and p57^{Kip2}) which interacts with all cyclin/cdks and the p16 family (*INK4*) which selectively inhibits the cdk4,6-mediated phosphorylation of pRb [105].

Disruption of this pathway is a prominent abnormality in both NSCLC and SCLC, albeit through different mechanisms. Variances in RB mRNA or protein expression in terms of absence, reduction in quantity, or alteration in function have been observed in this neoplasm [106–109]. The function of pRb is inactivated in more than 90% of SCLCs [110] as a result of different mechanisms including point mutations and abnormal mRNA expression [111]. Changes in the other pocket proteins (p107 and p130) have been detected in a minority of cases [112]. In contrast to SCLC, the majority of NSCLC cases exhibit abnormalities in the upstream regulators of the pRb pathway, including inactivation of p16 [113, 114] through different mechanisms [115], reduced levels of p27^{Kip} [116, 117], and enhanced expression of cyclin D1 [118]. It is likely that inactivation of cdk4,6 inhibitors (p16) and overexpression of cyclin D1 bypass the pRb checkpoint allowing progress through G1 into DNA synthesis [119, 120]. Immunohistochemical analyses of the RB gene product expression have been performed in malignant tissues from this human neoplasm [121]. Because the three members of the retinoblastoma protein family exhibit different growth suppressive properties, suggesting that they are not fully functionally redundant, our research group investigates their pattern of expression in large group of specimens of lung cancer, using an immunohistochemical approach. These Rb-family members displayed distinctive patterns when compared and contrasted with the different parameters. The highest percentage of undetectable levels in all the specimens examined and the tightest inverse correlation (*P* value) with the histological grading and with PCNA expression in the most aggressive tumor types were found

for pRb2/p130, which may suggest an important role for this protein in the pathogenesis and progression of lung cancer [122].

The tumor suppressor protein p53 also regulates progression through the G1 checkpoint of the cell-cycle. In particular, p53 is activated in response to DNA damage and serves to arrest cell-cycle progression in G1 and hence allow time for DNA repair. The fundamental importance of p53 in lung cancer is highlighted by the frequency of its mutations, 80% in SCLC and 50% in NSCLC [123]. It is recognized that p53 is a point of convergence of a complex network of signaling pathways that regulate its level in the cell. In turn, p53 binds to specific DNA sequences and transactivates a group of target genes (including the cell-cycle inhibitor *p21Waf1/Cip1*), thereby inhibiting cell proliferation and promoting apoptosis. Recent developments in this area have focused on the identification of p53-related genes such as *p73* [124] and in the elucidation of their role in lung cancer [125, 126]. In Figure 2, exemplificative immunostaining in non-small-cell lung cancers for the retinoblastoma proteins family and for p53 are depicted.

While several of the factors involved in regulating cell-cycle control have been investigated in lung cancer, few studies have examined multiple factors in the same tumor series. Our research group recently sets up a study to evaluate the expression of p53, p21, p16, and PCNA proteins in a large series of non-small-cell lung cancers (NSCLCs) to assess the integrity of cell-cycle checkpoints in these tumors, to evaluate the coexpression of these proteins, and, finally, to examine the relationship between these cell-cycle regulators and the clinicopathological features of NSCLCs, including their ability to predict survival in NSCLC patients [127]. When we looked at the correlation between clinicopathological data and expression of cell-cycle proteins, we found a negative correlation between lymph nodes status and p21, and p16 expression, suggesting a possible role for these two proteins in the progression of the disease. Interestingly, no correlation has been identified between p16, p21, and p53 expression. When we investigated by univariate analysis the correlation between different protein expressions and survival, we found that all the cell-cycle markers analyzed except for PCNA had a statistically significant correlation with survival. This result is in agreement with numerous data published about the cell-cycle checkpoints investigated in this paper and lung cancer [128–132]. Surprisingly, when we performed multivariate analysis, the only immunohistochemical parameter that resulted to influence overall survival was p16. This result is in agreement with the proposed hypothesis that the great majority of lung cancer samples have inactivated the RB/p16 tumor suppressor pathway. Among the clinical parameters, tumor staging was the only factor to influence survival in multivariate analysis. Finally, we grouped the lung cancer specimens based on p21 and p16 status. Interestingly, we found that the group of lung cancer specimens having both p21 and p16 negative displayed a significant shorter overall survival. Numerous data from the literature suggest the existence of a functional collaboration between distinct CDK inhibitor genes [133]. Indeed, it has been recently demonstrated that cell-cycle inhibition by p16 is associated

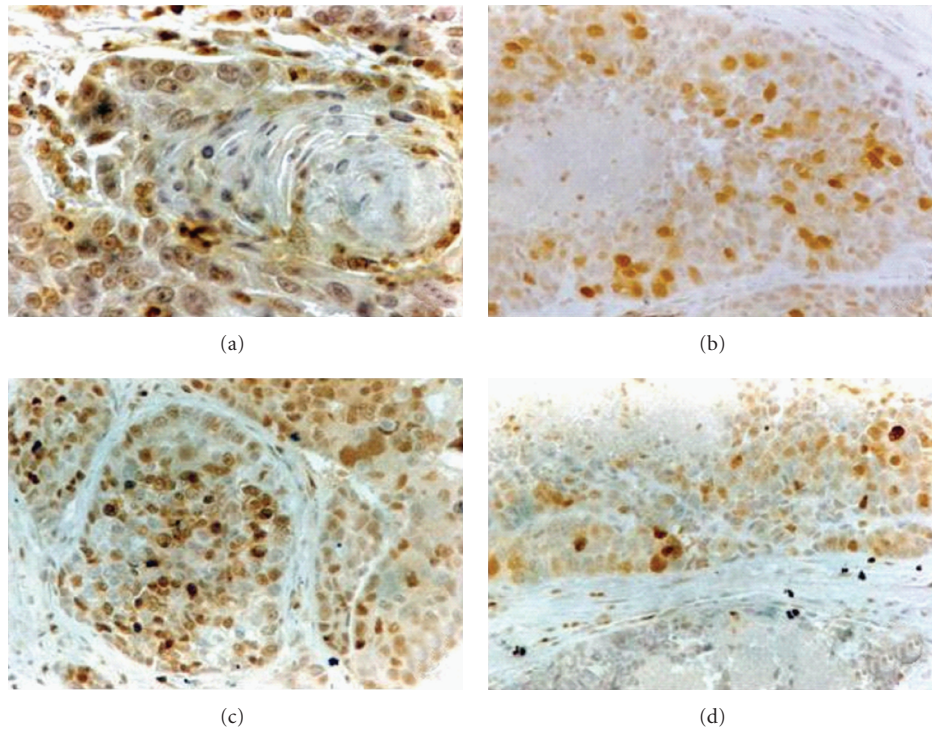


FIGURE 2: The immunohistochemical expression of retinoblastoma protein family and p53 in non-small-cell lung cancer. Exemplificative staining for Rb (a), p107 (b), p130 (c), and p53 (d) are depicted.

with a posttranscriptional induction of p21 and a strong inhibition of cyclin E-cdk2 kinase activity [134]. Moreover, it has been shown that members of the p21 family of proteins promote the association of D-type cyclins with CDKs by counteracting the effects of p16 molecules [135]. It has been, therefore, proposed that functional cooperation between different cell-cycle inhibitor proteins constitutes another level of regulation in cell growth control and tumor suppression. Taking into account the complicated functional network constituted by the cell-cycle regulator proteins, it appears evident that knowledge of the level of expression of these factors, and their coregulation, may be important in predicting patient clinical response to therapy.

In a different study, we determined the prognostic role of PCNA, p53, p27, pRb/p105, pRb2/p130, Cyclin D1, and p16 expression in a well-defined set of patients who underwent radical surgical treatment for non-small-cell lung cancer and had long-term followup [136]. Moreover, we explored the association of molecular markers with pathologic and clinical characteristics of this lung cancer population. The availability of the expression status of all tumor markers in the same set of patients provided a unique opportunity to determine whether alterations in p53, p27, pRb/p105, pRb2/p130, Cyclin D1, and p16 expression exert a cooperative or synergistic effect on lung cancer progression, metastasis, and survival. Surprisingly, when we performed multivariate analysis, the only immunohistochemical parameters that resulted to influence overall survival were p16, Cyclin D1, and pRb2/p130. Moreover, we showed that simultaneous loss of expression of three of these factors, the cyclin D1,

the cyclin-dependent kinase inhibitor p16, and the tumor suppressor pRb2/p130, identified a group of patients with worse prognosis. This result is in agreement with the proposed hypothesis that the great majority of lung cancer samples have inactivated the p16/Cyclin D1/retinoblastoma tumor suppressor pathway [137]. Our results demonstrate detection of an aberrant p53 in a discrete number of the specimens, which, however, does not correlate with patient survival in multivariate analysis. This finding contrasts with a previous study from our group which reported on 61 non-small-cell lung cancers and does not clarify the still debated prognostic role of p53 in lung cancer patients [10]. A recent study aimed to qualitatively review the association between p53 alterations and patient outcome by analyzing data from published papers, through a meta-analysis, showed that p53 mutation is a significant marker of poor prognosis in patients with lung adenocarcinoma [138]. Finally, we grouped the lung cancer specimens based on Cyclin D1, pRb2/p130, and p16 status. Interestingly, we found that the group of lung cancer specimens having three adverse prognostic factors displayed a significant shorter overall survival. Numerous data from the literature suggest the existence of a functional collaboration between distinct CDK inhibitor genes [139]. It has been therefore proposed that functional cooperation between different cell-cycle regulator proteins constitutes another level of regulation in cell growth control and tumor suppression [140].

4.2. Components of G2 and M Phases in Lung Tumors. High levels of cyclin B1 are observed in NSCLC [141]. Cyclin B1

has also been reported as a significant prognostic factor in NSCLC in multivariate analysis, suggesting that cyclin B1 expression may be a prognostic marker for these patients. CDK1 activity is controlled by phosphorylation, and this process is regulated by the WEE1 and PLK1 kinases. Indeed, downregulation of WEE1 expression has been reported in lung tumors [141]. By contrast, elevated levels of PLK1 are observed in NSCLC and overexpression of PLK1 is a negative prognostic factor in NSCLC patients [142]. Overexpression of *Aurora A* transcript and protein has been reported in NSCLC and was correlated with poor differentiation [143]. Although less extensively studied than *Aurora* genes, other mitotic genes display lung cancer-associated altered expression. They include microtubule-associated proteins such as TPX2 and TACC3 whose overexpression has been associated with poor clinical outcome [144].

4.3. Components of Cell-Cycle Checkpoints in Lung Tumors. CHFR, a mitotic checkpoint gene that delays chromosome condensation in response to microtubule poisons, has been described mutated and methylated in NSCLC [145]. Somatic mutations of ATM that correlate with smoking history and the presence of DNA repair defects are also detected in NSCLC [146]. Consistently, downregulation or absence of CHK2 expression has been reported in NSCLC, mainly due to hypermethylation of the *CHK2* gene promoter [147]. Defects in the SAC can lead to premature separation of sister chromatids and could facilitate chromosomal instability, which may favor tumor progression. Somatic mutations of several SAC regulators, such as *Bub1* and *Mad1*, have been reported in lung tumors, but the effect of these mutations on mitotic checkpoint signaling has not been examined [148, 149]. Finally, RASSF1A, a key negative regulator for mitosis progression and well-known tumor suppressor, undergoes frequent tumor-specific epigenetic inactivation in a wide range of tumors, and especially in lung cancer [150].

5. Conclusion

In conclusion, all the works mentioned here provide useful information on the prognosis of newly diagnosed cases of lung cancer and would allow researchers to recognize a subgroup of patients with significantly improved survival, in which it could be possible to achieve better response to therapy. Nevertheless, targeting multiple checkpoint proteins may represent a good therapeutic strategy for the development of new molecular treatments for lung cancer. The data presented in this paper support this hypothesis and strongly suggest further works aimed at investigating the simultaneous expression of numerous cell-cycle regulators in lung cancer.

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