Prognostic and Predictive Value of Cell Cycle Deregulation in Non-Small-Cell Lung Cancer

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Key Words

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Abstract

Non-small-cell lung cancer (NSCLC) is among the most frequently diagnosed malignancy and a leading cause for cancer mortality worldwide. Despite various efforts, practical prognostic and predictive markers are still few. We review recent findings concerning the cell cycle in NSCLC and discuss prognostic and predictive aspects as well as the challenge of targeted therapeutic approaches. Deregulation of the cell cycle is a common event in NSCLC. Usually, several defects of cell cycle regulation are concomitant and have a cumulative adverse effect on prognosis. Therefore, analysis of a variety of interacting molecules is desirable for adequate deductions. Immunohistochemical interpretations should include the subcellular staining localization, since this can reflect the functional properties of a protein. Overexpression of cyclins, especially D-type cyclins, has repeatedly been associated with poor prognosis in NSCLC. Predictive data is less conclusive; however, loss of the expression of cyclin-dependent kinase inhibitors seems to correlate with sensitivity to antiproliferative drugs. Various inhibitors of Aurora kinases are currently being evaluated regarding their

potential as targeted therapies in NSCLC. In conclusion, the cell cycle offers several prognostic, predictive and therapeutic possibilities in NSCLC, many still developmental. Progress in this field has the potential to improve the current scenario for NSCLC patients.

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Epidemiology and Risk Factors

A recent overview on global cancer statistics showed that lung cancer was the most commonly diagnosed cancer, as well as the leading cause of cancer death (fig. 1) [1]. The association between cigarette smoking and lung cancer is one of the most thoroughly investigated medical issues, with compelling evidence indicating that smoking is the predominant causal factor for lung cancer [2–5]. Variations between countries and genders in the incidence of lung cancer largely reflect differences in the stage and degree of the tobacco epidemic [6, 7]. Other known risk factors for lung cancer include exposure to occupational and environmental carcinogens such as asbestos, arsenic, radon and polycyclic aromatic hydrocarbons as well as preexisting lung diseases (e.g. tuberculosis and pneumonia) [7, 8].

Fig. 1. Top 5 cancer incidences and deaths worldwide in 2008 [1].

Histology

Approximately 80% of all malignant lung tumors are classified as non-small-cell lung cancers (NSCLC) representing a heterogeneous group [9]. Around 20% are small-cell lung carcinomas (SCLC) and less than 1% are nonepithelial tumors (e.g. lymphomas, sarcomas, germ cell tumors or melanomas). The most common NSCLC subtypes are squamous-cell carcinomas (SCC) and adenocarcinomas (ACA). SCC are predominantly linked to smoking, tend to arise centrally in the lung, have the capacity to grow to large sizes and are proportionally more common in male patients [10, 11]. In contrast, ACA are usually found in the periphery of the lung and are proportionally more frequently diagnosed in women; although they are the most common type of lung cancer observed in nonsmokers, they have been increasingly associated with smoking in recent years [11–13]. ACA represent a very heterogeneous entity, obvious by the many morphological subtypes and various (often

mutually exclusive) genetic alterations. This circumstance has recently prompted a new multidisciplinary classification approach for ACA [14]. SCLC have a greater potential to metastasize than other types and are generally associated with a very poor prognosis [15]. Almost all patients suffering from SCLC are current or former smokers [15].

Symptoms, Prognosis and Management

In the last decades, the overall prognosis for patients with lung cancer has remained dismal worldwide, with 5-year survival rates averaging between 6 and 14% for men and between 7 and 18% for women [7]. The main reason for this poor outcome is that lung cancer is typically asymptomatic in the early stages of its development or may only cause nonspecific symptoms [16]; thus, the majority of lung cancer patients are diagnosed in a more advanced disease stage.

Surgical resection is the treatment of choice for early localized tumors, whereas for advanced lung cancer treatment options become limited [16–19]. Trials involving platinum-based doublets have shown that adjuvant chemotherapy improves 5-year overall survival by 8 to 15% in selected patients with resected stages II and IIIA NSCLC [20–22]. Adjuvant chemotherapy is currently still not routinely recommended for stage I disease, although trials have shown benefits regarding overall and relapse-free survival [23]. Taken together, some early-stage patients will have disease recurrence despite surgical resection with curative intent and other patients in an advanced stage may receive unnecessary adjuvant chemotherapy. These observations render NSCLC a very heterogeneous disease, calling for improved patient stratification regarding prognostication of survival time and prediction of treatment response.

Prognostic markers are defined as parameters (patient- or tumor-associated) that predict survival outcome independent of treatment. Predictive markers on the other hand are parameters for predicting treatment outcome, and may refer to response or survival. To date, the clinicopathologic staging system has been the standard for determining prognosis, although its value for the individual patient is questionable [24]. In ACA, additional mutation analysis of the epidermal growth factor receptor gene (*EGFR*), and – if unmutated (wild-type) – of the presence of the echinoderm microtubule-associated protein-like 4 (*EML4*) anaplastic lymphoma kinase 1 (*ALK1*) fusion gene or *ALK1* gene rearrangements, have therapeutic implications [25, 26]. Patients with tumors harboring the *EML4-ALK1* fusion gene or activating mutations in the *EGFR* gene are likely to respond to respective tyrosine kinase inhibitors (TKI) [25, 26]. For approximately 40% of all ACA and for virtually all of the other histologic NSCLC subtypes, there are currently no predictive molecular parameters available [27]. To improve individualized treatment, many studies are focusing on the identification of prognostic and predictive molecular markers. A variety of oncogenes, tumor suppressor genes, cell cycle modulators and molecules related to tumor invasion, metastasis and angiogenesis have been reported in this regard [28–32].

Deregulation of the cell cycle can lead to uncontrolled cell proliferation and it has been found in many different human malignancies [33]. Molecules involved in the cell cycle have often been reported as prognostic markers and although the cell cycle also seems to offer potential anti-cancer targets, such strategies are not yet routinely established [34–37]. This review summarizes the importance

Fig. 2. Schematic of the major regulators of the cell cycle. Cyclin D/cyclin-dependent kinase 4 (CDK4) and cyclin D/CDK6 phosphorylate the Rb which then releases E2F transcription factors leading to expression of cyclin E. Cyclin E/CDK2 causes transition from the G1 to the S phase. Cyclin A/CDK2 drives the cell cycle from the S phase to mitosis and cyclin B/CDK1 conveys progression through the M phase. The inhibitory function of the CDK inhibitors (CKI) p16, p21, p27 and p57 is depicted by bars. Transcription of p21 is induced by p53.

and recent findings concerning the cell cycle in NSCLC and discusses the prognostic and predictive aspects as well as the challenge of therapeutic approaches.

Cell Cycle Regulation

The major regulators of the cell cycle are depicted in figure 2. The mammalian cell cycle is composed of 5 phases. Quiescent (nondividing) cells are in the G0 phase (interphase). In response to mitogenic signals, cells enter the G1 phase, which is characterized by an intracellular increase of D-type cyclins (D1, D2 and D3), resulting in cyclin D/cyclin-dependent kinase 4 (CDK4) and cyclin D/CDK6 complexes. These cyclin/CDK complexes initiate phosphorylation of the retinoblastoma proteins (RB), which leads to the release of E2F transcription factors, allowing expression of cyclin E [38]. Cyclin E binds and activates CDK2, further phosphorylating RB and causing the transition from the G1 to the S phase [39]. The G1/S transition is also known as the restriction point, and is the critical point of the cell cycle. After passing this point, cells are committed to the next phases. In the S phase, a copy of the genetic material is produced, which must then be equally divided among the two daughter cells. CDK2 is activated by cyclin A to drive the cell cycle

Fig. 3. Fluorescence in situ hybridization analysis of the *Cyclin D1* (*CCND1*) gene with dual-colored break-apart probes flanking *CCND1* (from Vysis/Abbott, Downers Grove, Ill., USA; *CCND1*: order No. 05J96-001). Note split red and green signals (red and green/black and gray arrows, in print and online, respectively) in a large-cell carcinoma corresponding to *CCND1* gene rearrangements as well as 1 fused yellow signal (yellow/white arrow, in print and online, respectively) corresponding to the nonrearranged allele.

from the S phase to mitosis, a stage termed G2 [33]. During the later S phase and early G2 phase, the centrosomes are duplicated and form the poles of the mitotic spindle, which give rise to dynamic microtubules [40]. These events are controlled by CDK1 activated by cyclin A [41]. After disintegration of the nuclear envelope, cyclin A is degraded and cyclin B/CDK1 complexes convey the progression through the M phase by promoting chromosomal condensation and assembly of the spindle apparatus [41]. The anaphase promoting complex or cyclosome (APC-C) degrades cyclin B and final segregation is facilitated [42].

Two families of CDK inhibitors (CKI) are known and play an important role in maintaining the cell cycle control [43]. The INK4 proteins, including INK4A (p16), INK4B (p15), INK4C (p18) and INK4D (p19), bind to CDK4 and CDK6, thus inhibiting their activation by D-type cyclins. The WAF/CIP/KIP family of proteins includes WAF1/CIP1 (p21), KIP1 (p27) and KIP2 (p57), which form heterotrimeric complexes with the G1/S CDK. Proper progression through the cell cycle is assisted by checkpoints that detect defects during DNA synthesis and chromosome segregation and modulate CDK/CKI activity. The cell cycle is then halted, providing time

to repair such defects. If the damage is irreparable, programmed cell death or senescence is induced. An important pathway involved in preventing cells from entering the S phase is the activation of the tumor suppressor gene *p53*, which induces transcription of *p21*.

Accurate control of the cell cycle is essential for sustaining adequate cell proliferation. On the other hand, a characteristic of cancer cells is uncontrolled proliferation, independent of growth factors or growth inhibitory signals and tolerance towards DNA defects [44]. These features are acquired by the accumulation of various mutations [45, 46]. The importance of cell cycle deregulation in cancer is underlined by an increasing amount of related literature. Numerous studies show that among the many altered pathways involved in lung carcinogenesis, cell cycle deregulation is a frequent and critical occurrence [40, 47, 48]. Zhu et al. [48] presented an impressive graph depicting the amount of publications referring to various cell cycle-related molecules and their prognostic significance in NSCLC. *p53*, Bcl-2, Ki-67, cyclin D1 and RB were among the most extensively reported parameters. The role of other CKI and non-D-type cyclins (especially cyclins A and B) as well as CDK is less clear. Interestingly, the prognostic values differ among reports for most markers, which may be a reason for their lack of utility in everyday practice [48].

Cyclins in NSCLC

D-Type Cyclins

Cyclins are among the most thoroughly investigated molecules involved in the cell cycle in NSCLC. This is especially true for D-type cyclins, most likely because they are responsible for the transition from the G1 to the S phase. Overexpression of D-type cyclins can shorten the G1 phase and reduce the cell dependency on mitogens [49–51]. Cyclin D1 is often both amplified/rearranged and overexpressed in NSCLC as well as in preinvasive tumorous lung lesions, indicating an early step in NSCLC carcinogenesis (fig. 3) [37, 52, 53]. The rate of gene amplification of the cyclin D1 locus (*CCND1*) has been reported at between 15 and 35% in NSCLC [37, 52, 53]. This discrepancy can be explained by the amount of gene copies that were required for considering *CCND1* as amplified, which varied between groups [37, 52, 53]. Overexpression is less variable and has been observed in around 40% of examined cohorts; importantly, the correlation between *CCND1* gene amplification and increased cyclin D1 protein expression is obvious in

NSCLC, and has also been found in other tumors [54, 55]. Although reports concerning cyclin D1 in NSCLC offer divergent conclusions regarding clinical outcome, most studies, and especially those published recently, link overexpression to worse prognosis [37, 56]. An important means for obtaining more informative data is the combined analysis of multiple molecules known to interact with each other, which is superior to single-marker studies for the prediction of clinical outcome in NSCLC [37, 57]. For example, Burke et al. [58] analyzed 106 surgically resected, predominantly early-stage NSCLC with regard to the protein expression of cyclin D1, p21, RB, p16 and p53, as well as the mutational status of *p53*. Overexpression of cyclin D1 and p21, loss of p16 and expression and mutation of *p53* were frequently observed [58]. No single marker was prognostically relevant, in contrast to certain combinations. Cyclin D1 overexpression in RB-negative tumors and cyclin D1 overexpression with either p53 expression or *p53* mutation were associated with significantly poorer prognosis [58]. In a similar study design, Myong [59] also observed that cyclin D1 overexpression, p16 loss and the inactivation of RB (by phosphorylation) are common in NSCLC. Furthermore, cyclin D1 overexpression correlated with p16 loss and RB inactivation and the overall survival of patients with the combination of cyclin D1 overexpression and loss of p16 tended to show a rapid decline [59]. Our own observations of a multitude of cell cycle markers in a comparably large cohort (405 cases) of surgically resected NSCLC revealed that cyclin D1 overexpression is associated with the overexpression of cyclin D3, p27 (nuclear), p21 and the loss of p16 [36, 37]. The only independent prognostic parameters by multivariable analysis were the overexpression of cyclin D1 and the loss of p16, both indicating poor overall survival [36, 37]. Taken together, these studies show various reproducible trends regarding NSCLC and cyclin (D)-associated cell cycle molecules: (1) deregulation of the G1 cell cycle phase is frequent and (2) it plays an important role in the carcinogenesis of NSCLC, (3) analysis of multiple functionally associated markers is more informative than single-parameter studies, (4) several defects of the cell cycle regulation are frequently concomitant and (5) they have a cumulative adverse effect on prognosis.

The special interest in cyclin D1 has led to the characterization of two alternative splice variants (cyclin D1a and D1b) with different coding sequences that may be related to clinical outcome in NSCLC [56, 60]. Difficulties in assessing this issue are due to the fact that cyclin D1 expression in NSCLC has mainly been analyzed immu-

nohistochemically [37, 60, 61] and most available antibodies detect total cyclin D1 or the C-terminus of cyclin D1a [62]. In addition, cyclin D1b is usually present in quantities below the sensitivity of available assays [63]. Li et al. [63] used real-time reverse transcription polymerase chain reaction and immunohistochemistry (including a new specific antibody against cyclin D1b) to evaluate messenger RNA (mRNA) and protein expression levels of total cyclin D1 and the splice variants D1a and D1b, all of which were significantly upregulated in NSCLC when compared to nonmalignant lung tissue. They found that although the absolute expression levels of cyclin D1a were higher than those of cyclin D1b, the relative expression ratios of cyclin D1b mRNA between malignant and nonmalignant lung tissues were higher than those of cyclin D1a mRNA [63]. In contrast to cyclin D1a, cyclin D1b mRNA expression correlated with histological grade, lymph node metastasis, distant metastasis and tumor stage [63]. Increased mRNA and protein expression of cyclin D1b were also associated with poor survival, the latter even being an independent risk factor [63]. These results indicate that the splice variant cyclin D1b, in particular, contributes to tumorigenesis and may be a better prognostic marker than cyclin D1a or total cyclin D1 in NSCLC.

Cyclins E, A and B

Cyclin E expression has commonly been associated with shorter overall survival for resectable NSCLC, although some studies found no prognostic value [37, 47]. As reported for cyclin D1, cyclin E is also often overexpressed in precursor lesions [64].

Compared to the numerous studies of regulators of the G1/S transition, cyclins of the S phase and G2/M transition are not well characterized in NSCLC [48]. Increased expression of cyclins A and B1 has been shown to correlate with poor tumor differentiation and squamous-cell histology in NSCLC [65–67]. Cooper et al. [65] recently reported frequent increases of cyclin A and B1 protein expression in stages I–II NSCLC (58.9 and 40.9%, respectively) as well as in precursor lesions, compared to normal bronchial epithelia. They found that overexpression of cyclin B1 was significantly associated with poor prognosis on univariate, but not multivariate analysis; this was in contrast to others reporting cyclin B1 as an independent prognostic factor [65, 66]. It is presently not clear if a cyclin B1-associated outcome is linked to a certain histological subtype, since this has been proposed for SCC as well as for ACA [65–67]. Cyclin A does not seem to be a prognostic marker for NSCLC, although this might be

restricted to early tumor stages. Analysis of stages I–III found cyclin A to be associated with poorer survival [65, 68–71].

Predictive Value of Cyclin Dereglulation in NSCLC

Although many reports concerning the prognostic significance of cyclins for NSCLC exist, information regarding predictive power is lacking. One study noted a downregulation of cyclin D1 by suppressing EGFR signaling in cell lines, suggesting that reduction of cyclin D1 expression may be a sensitive marker for the response to TKI [72]. They also found that cyclin D1 is overexpressed in lung cancer cells harboring mutant *EGFR*, which is in line with studies reporting that mutant *EGFR* preferentially activates AKT and STAT pathways, both implicated in *CCND1* gene expression [73–77]. Flavopiridol, a drug that directly inhibits CDK4 and CDK6 and reduces the transcription of cyclin D1 by inhibiting CDK9 and CDK7, was shown to deplete cyclin D1 and induce substantial apoptosis in cell lines harboring *EGFR* mutations [78]. Importantly, TKI-resistant cells remained sensitive to flavopiridol, which may be a strategy to overcome/prevent resistance to EGFR inhibitors in patients with *EGFR*-mutant NSCLC [72].

Cyclin/CDK complexes normally translocate to the nucleus and this is usually where the in situ immunohistochemical expression of cyclins is detected [37]. Many studies even disregard the cytoplasmic staining of cyclins, as it is considered nonspecific [59, 63]. Interestingly, in some tumors including NSCLC, cyclin B1 is predominantly observed in the cytoplasm [65, 79, 80]. Cyclin B1/CDK complexes are thought to remain in the cytoplasm due to DNA damage [81]. Therefore, it may be important to note the exact subcellular immunohistochemical staining pattern of cell cycle-related molecules, rather than disregard certain results. This issue is discussed in greater detail for CKI in the following section.

Cyclin-Dependent Kinase Inhibitors

p16

The first tumor suppressor gene found in lung cancer was *RB* [82]. Although loss of the RB protein (pRB) is reported in only around 15% of NSCLC, most NSCLC have RB inactivation due to upstream regulatory defects [40, 83–85]. The CKI p16 inhibits cyclin D-dependent phosphorylation of the pRB by binding CDK4 and CDK6. Loss of p16 leads to phosphorylation of RB, releasing cell cycle inhibition and allowing uncontrolled

G1/S progress. Loss of p16 has been described for a variety of tumors including NSCLC and is usually associated with worse prognosis [86]. We (and others) have found loss of p16 expression to be an independent predictor of poor overall survival [36, 87, 88]. Major mechanisms of decreased p16 activity include gene deletion and hypermethylation of the CpG island promoter region, both being observed in NSCLC, whereas point mutations are rare [89–91]. Loss of p16 protein expression by immunohistochemistry is known to be an accurate method for detection of *p16* gene inactivation events [91]. Our observations show that loss of p16 protein expression is frequent in NSCLC (63%) and is an especially common feature of SCC compared to ACA, which is well in line with most previous studies addressing this issue [59, 92]. Since most SCC are p16-negative, p16-positive SCC with a nondeleted *p16* gene might represent a distinct biological group. Considering that there may be a connection with human papilloma virus (HPV) infection (e.g. as is known for cervical cancer) in SCC expressing p16, we analyzed this group of tumors regarding a possible viral association [36]. Notably, nearly all of the patients were men (8/9) and the median overall survival time was 73 months compared to 44.7 months for the entire SCC cohort [36]. Tumor stage and grade, Ki-67 index and smoking status did not considerably differ [36]. We also performed immunohistochemistry using the Cytoactiv HPV L1 screening set (detection of HPV L1 capsid protein) as well as the Cytoactiv HPV L1 high-risk set (detection of L1 capsid protein of the high-risk HPV subtypes: 16, 18, 31, 33, 35, 39, 45, 56 and 58) (both from Cytoimmun Diagnostics GmbH, Pirmasens, Germany) [36]. No case showed a positive nuclear staining signal; thus, as expected, prognosis was indeed better, although no indication of HPV infection was apparent [36].

It has been shown that expression of p16 and cyclin D1 inversely correlate with each other, which reflects their functions in the cell cycle [59]. Since p16 inhibits CDK, which becomes active when bound to cyclin D1, loss of p16 protein can lead to elevated levels of cyclin D1 protein. Accordingly, the combination of cyclin D1 overexpression with loss of p16 has been considered useful to predict reduced overall survival prognosis [93].

DNA methylation plays an essential role in the maintenance of genomic stability; however, alterations in methylation patterns frequently occur in tumor cells [94]. Hypermethylation in the promoter regions of tumor suppressor genes are commonly associated with epigenetically mediated gene silencing [95]. In lung

cancer, *p16* gene hypermethylation has been detected in 17 to 84% of cases in a smoking habit-dependent manner and may be a candidate marker for prediction and prognostication in NSCLC [96, 97]. Studies have demonstrated that aberrant *p16* promoter hypermethylation is an early and critical event in the development of NSCLC [98]. Gene promoter hypermethylation has even been reported as a molecular marker for identifying healthy individuals at a high risk for cancer incidence since it has been observed in the serum and sputum of chronic smokers without clinical disease [99]. The importance of gene promoter hypermethylation lies in the possibility of epigenetic treatment options. Epigenetic changes involved in cancer development, unlike genetic changes, are reversible. Thus, patients with tumors harboring epigenetic alterations could benefit from treatment with demethylating agents. 5-Azacytidine is a demethylating agent that inhibits DNA methyltransferase 1 in replicating cells [100] and has recently been shown to prolong survival and improve quality of life in patients with myelodysplastic syndromes, while maintaining a favorable adverse-effect profile [101]. Prospective studies in NSCLC with promising results are ongoing [102]. A recent interim analysis of a phase II trial has reported that the combination of azacytidine and entinostat (a histone deacetylase 1 and 3 inhibitor) has a durable benefit in patients with advanced relapsed NSCLC [102]. So far, predictive markers for response to demethylating agents have not been established; however, the *p16* methylation status may be a good candidate. The frequency and type (homozygous/heterozygous) of deletions of the *p16* gene locus varies in the literature [36, 91]. We recently performed a study examining the relationship between the protein expression, gene status and promoter hypermethylation of *p16* [36]. Our data suggest that hypermethylation of the promoter region of *p16* is most likely the initial step toward loss of p16 function in NSCLC, followed by heterozygous deletion, which finally results in complete loss of protein expression; in our large series, we did not observe any cases of homozygous *p16* deletion. Considering the possibility of early detection of hypermethylated gene regions, this data may lead to the identification of patient subgroups more likely to benefit from upcoming epigenetic treatment strategies.

The other members of the INK4 CKI family are not well characterized in NSCLC. *p15* has been reported as frequently inactivated by homozygous deletions in NSCLC together with *p16* since both loci cluster together [103–105]. Methylation of the *p15* gene has been found,

but its effect on protein expression is poorly investigated [105–107]. *p18* and *p19* have been examined in only very few studies in NSCLC [108, 109]. Polymorphisms have been found in the *p18* and *p19* genes, the former being associated with a decreased risk of death [109].

p21

p21, of the WAF/CIP/KIP family of CKI, is an inhibitor of several cell cycle phases, inhibiting cyclin D1/CDK4 and cyclin E/CDK2 complexes in the G1 phase and cyclin A/CDK2 complexes prior to the G2/S transition. Expression of *p21* has been noted more commonly in early-stage NSCLC and seems to be a predictor of improved prognosis, although controversial reports exist [110–112]. For patients with nondetectable *p21* and *p16* expression, overall survival has been reported as significantly decreased compared to cases with loss of only one CKI [87], emphasizing the cumulative effect of cell cycle deregulation mechanisms on adverse outcome. Recently, promoter hypermethylation of *p21* was described in 30% of NSCLC and suggested as a critical mechanism for *p21* inactivation, especially in ACA [113]. Although DNA methylation was a frequent finding, loss of the *p21* gene product was more frequent, indicating that this epigenetic modification is not the main mechanism of *p21* suppression [113]. Mutations of the *p21* gene have rarely been documented in malignancies [114]. Absence of *p21* expression has been associated with resistance to cisplatin, one of the most potent anticancer agents and most effective systemic chemotherapies for NSCLC [115–117]. It has recently been shown that by increasing *p21* expression through upregulation of *p21* gene expression in an NSCLC cell line, chemosensitivity to cisplatin was enhanced and cell growth was significantly inhibited in vivo and in vitro [118]. RNA-induced gene activation, a transcriptional gene activation phenomenon targeting gene promoter regions, was applied in this study, suggesting that this technique in combination with cisplatin-based chemotherapy may increase effectiveness, especially in drug-resistant tumors [118].

p53, often termed the guardian of the genome, is an important player in a main DNA damage response pathway [33]. Upon DNA damage, the tumor suppressor gene *p53* is activated leading to transcriptional activation of *p21*, thus preventing transition to the S phase [119]. In the presence of an altered form or loss of the *p53* protein, *p21* expression is drastically reduced or nondetectable [113]. Mutations of the *p53* gene are frequently found in NSCLC [120] and *p53* nuclear immunoreactivity in tumors has been regarded as a surrogate marker for the presence of a

p53 gene mutation, since mutant *p53* protein has a longer half-life than the wild-type protein [121]. On the other hand, sensitivity and positive predictive value of immunohistochemistry for *p53* mutational status varies in the literature [120, 122]. Generally, *p53* gene mutations and *p53* protein overexpression are seen as weak prognostic markers of poorer outcome in NSCLC [123–126]. Conclusive predictive data concerning *p53* in early-stage NSCLC treated with adjuvant chemotherapy is sparse. An Eastern Cooperative Oncology Group study randomly assigned completely resected stage II–IIIA NSCLC patients to receive adjuvant radiotherapy with or without etoposide plus cisplatin and found that *p53* gene mutation and *p53* protein overexpression were neither prognostic of poorer survival nor predictive of a benefit from chemotherapy [127]. Another group, using tumor samples from JBR.10, a North American phase III intergroup trial that randomly assigned patients with completely resected stage IB and II NSCLC to receive 4 cycles of adjuvant cisplatin plus vinorelbine or observation alone, found that *p53* protein overexpression was both prognostic for poorer survival and predictive of a greater survival benefit from adjuvant chemotherapy [120]. The observation that *p53* protein expression rather than *p53* mutation is linked to a more aggressive clinical outcome has been speculated to be linked to the activation of oncogenic pathways regardless of *p53* mutational status leading to a stable (detectable) *p53* protein [128–130]. The effect of *p53* mutations and aberrant *p53* expression on the sensitivity of anticancer agents is contradictory [120, 129, 131]. Sensitivity to cisplatin could possibly be caused by the absence of downstream activation of *p21*, leading to failure of DNA repair [131]. The retained ability to sufficiently repair DNA defects is believed to diminish the survival benefit from platinum-based chemotherapy in patients, whose tumors have high expression of the excision repair cross-complementation group 1 (ERCC1) protein [132]. The reported benefit from adjuvant chemotherapy in patients with low expression of ERCC1 leads to the conclusion that the best efficacy of platinum-based chemotherapy in NSCLC may be seen when ERCC1 expression is low and *p53* expression is high and *p21* inactive [120].

Due to the availability of tumor tissue, nearly all molecular studies are performed on surgically resected (i.e. generally early-stage) tumors. Patients in TNM tumor stage IIIA with metastasis to the ipsilateral mediastinal lymph nodes (N2) represent a heterogeneous group with regard to prognosis and treatment [133]. There is no consensus on the preferred management of patients with pre-

operatively diagnosed N2 disease, although technically they are considered potentially resectable [134]. A study aiming to identify characteristics for deciding whether or not to perform surgery on patients with N2 disease focused on molecules of cell cycle regulation [133]. By examining the expression of proteins involved in the RB and *p53* pathway, they found the loss of *p16* and *p21* to be independently associated with poorer overall survival [133]. Again, an additive functional cooperation between different cell cycle proteins was obvious, since patients with expression of both *p16* and *p21* had the best prognosis (compared to patients expressing only one or neither marker) [133]. They concluded that preoperative patients with N2 disease and expression of both *p16* and *p21* in their primary tumors are expected to have favorable post-operative survival outcome and may be candidates for primary resection [133]. Although the study limitations included a small sample size (61 cases) and a retrospective nature, the possibility of cell cycle regulators facilitating the decision process for surgical tumor resection arises. This is an intriguing approach, particularly considering the increasing use of minimal-invasive mediastinal lymph node tissue sampling (endobronchial ultrasound-guided, transbronchial needle aspiration).

p27

In addition to the cyclin/CDK complexes inhibited by *p21*, the CKI *p27* is also able to inhibit cyclin D/CDK6. The importance of *p27* is illustrated by knock-in mice expressing mutant *p27* protein unable to bind and inhibit cyclin/CDK complexes. These mice have increased stem and progenitor cell populations as well as a variety of neoplasms [135]. Reduced protein levels of *p27* have been reported in NSCLC and usually correlate with poor overall survival [136, 137]. The International Adjuvant Lung Cancer Trial demonstrated that adjuvant cisplatin-based chemotherapy improves the 5-year survival rate of patients with completely resected NSCLC, a benefit that was confirmed by a pooled analysis of similar studies (survival improved at 5 years by an absolute value of 5%) [20, 138]. To determine whether cell cycle regulators are of prognostic or predictive value for NSCLC patients, a study was performed analyzing tumor specimens from the International Adjuvant Lung Cancer Trial regarding protein expression of *p16*, *p27*, cyclins D1, D3 and E and Ki-67 [139]. It was shown that patients with *p27*-negative tumors benefited from cisplatin-based chemotherapy, whereas patients with *p27*-positive tumors did not [139]. Previous in vitro studies have indeed implicated *p27* in drug resistance [140]. Since most anticancer agents pref-

entially target rapidly dividing cells, a low proliferation fraction is a major factor of drug resistance [141]. By preventing the G1/S transition, p27 halts the cell cycle, thus providing protection from antiproliferative drugs. Furthermore, p27 has an antiapoptotic effect; it inhibits the activation of procaspases and cytochrome C release, which may also contribute to a lack of chemosensitivity [142].

Generally, immunohistochemical detection p27 is associated with improved survival outcome and loss of p27 correlates with poor survival in a variety of cancer types [143, 144]. On the other hand, studies involving lymphomas, in particular, have demonstrated that highly malignant neoplasms may also be associated with increased staining of p27 [145, 146]. High levels of p27 expression have also been found in some breast cancer cells [147]. An explanation for such conflicting data may be connected to the exact subcellular localization of p27 in tumor cells (fig. 4). Various manufacturers of antibodies directed against p27 depict positive results as a nuclear staining signal. Indeed some studies have only regarded p27 immunohistochemistry as positive when nuclear staining was observed, irrespective of cytoplasmic presence [57, 139]. This interpretation may not accurately reflect the biological importance of p27. As discussed, the INK4 CKI are frequently lost by deletion, promoter hypermethylation or mutation, events that are extremely rare in the *p27* gene [148]. Nonetheless, p27 has been termed a tumor suppressor protein, since its inactivation has been linked to tumorigenesis [149]. Mechanisms of inactivation include loss of p27 expression as well as nuclear exclusion [150]. Degradation of the p27 protein by proteolysis is thought to be the major pathway for reducing its abundance [150]. In a first step, p27 is phosphorylated by cyclin E/CDK2 complexes (at threonine 187), followed by the ubiquitin-dependent proteasome degradation of p27 [151–155]. In this way, proteins intended for degradation are tagged with the small protein ubiquitin by ubiquitin ligases, resulting in polyubiquitin chains which are then bound by proteasomes leading to proteolysis of the target protein [151–155]. In response to mitogenic stimulation, the human kinase interacting stathmin may also phosphorylate p27, however at a different residue site (serine 10), leading to nuclear export of the p27 protein [156]. The protein kinase B (PKB)/AKT is responsible for p27 phosphorylation at threonine 157, inducing cytoplasmic retention [157]. PKB/AKT also phosphorylates the forkhead transcription factor Afx, thus keeping it in the cytoplasm and preventing *p27* gene transcription [158, 159]. Cytoplasmic localization of p27 does not result in its degrada-

Fig. 4. Schematic of the major p27 functions. In the nucleus cyclin D/cyclin-dependent kinase 4 (CDK4) complexes titrate p27 away from cyclin E/CDK2 complexes, thus facilitating activation of cyclin E/CDK2. Cyclin E/CDK2 complexes phosphorylate p27 leading to its degradation in the nucleus. Human kinase interacting stathmin (hKIS) phosphorylates p27 leading to its nuclear export. Cytoplasmic p27 can inhibit cytochrome C and procaspases leading to apoptosis. Protein kinase B (PKB)/AKT phosphorylates cytoplasmic p27 inhibiting its nuclear transport. PKB/AKT also phosphorylates the p27 transcription factor Afx, inhibiting its nuclear entry and thus preventing *p27* transcription.

tion [160]. In addition, cyclin D/CDK4/6 complexes sequester p27, serving to titrate p27 away from cyclin E/CDK2 complexes, which is important for CDK2 activation [161, 162]. Thus, sequestration as well as the export of p27 from the nucleus to the cytoplasm facilitates activation of cyclin E/CDK2, which maintains low levels of p27 by initiating p27 degradation. There is evidence that p27 is a major target of tyrosine kinase receptors including EGFR and ErbB2/Her2, resulting in cancer cell proliferation by (1) decreasing p27 levels, (2) sequestering p27 away from cyclin E/CDK2 complexes and (3) causing nucleus-to-cytoplasm transport (nuclear exclusion) of p27 [163–165]. The question arises if cytoplasmic relocalization of p27 is only a means of abrogating its function, or if cytoplasmic p27 has additional effects. For example, it is known that p21 not only inhibits CDK4, but when present in the cytoplasm it can inhibit caspase-3 by interacting with procaspase-3, thus inhibiting apoptosis [166]. In analogy, cytoplasmic p27 has been shown to prevent

Fig. 5. Immunohistochemical stains for p27. **a** An SCC with p27 expression only in the nucleus. **b** An SCC with nuclear and cytoplasmic expression of p27. **c** A large cell carcinoma with p27 expression only in the cytoplasm. Taking figure 4 into consideration, these different subcellular localizations of p27 are most likely of different, probably biologically completely opposite, functional importance.

cytochrome C release and also inhibits other procaspases [142]. This indicates that p27 can inhibit apoptosis upstream of mitochondrial function (cytochrome C) as well as downstream where caspases are involved. Supporting the notion of p27 acting as an oncoprotein, studies have linked high p27 expression to higher grades of endometrioid cancer and poorer prognosis [167]. Furthermore, tumors with cytoplasmic expression of p27 are associated with a more aggressive clinical course compared to p27 localized to the nucleus [168, 169]. Such dual functions of a protein are not a new phenomenon. Another example of a protein that plays at least two distinct roles, depending on nuclear or cytoplasmic localization, is β -catenin (regulator of transcription and cell adhesion molecule) [37]. Accordingly, one may conclude that the combination of high nuclear and low cytoplasmic expression of

p27 should confer the best prognosis. Indeed, a recent study has reported this correlation for high-grade astrocytomas [169]. Our own observations show that when taking the exact subcellular immunohistochemical staining pattern into account, cytoplasmic and nuclear p27 expression strongly correlate with each other in NSCLC (fig. 5) [37]. In contrast to other reports, overexpression of nuclear p27 was associated with decreased overall survival [37, 48]. A likely explanation for this controversial finding is the association of p27 with cyclin D, which sequesters p27 in the nucleus, preventing its inhibition of cyclin E/CDK2 complexes. Indeed, our results demonstrated a significant correlation between increased nuclear p27 expression and increased expression of the cyclins D1, D3 and E [37]. In addition, the increased expression of these cyclins was also associated with increased ex-

pression of p21 [37]. These findings emphasize that immunohistochemical stainings of p27 (and p21) do not necessarily reflect its supposed functions. For additional information, the exact subcellular localization and association with other cell cycle proteins must be taken into account.

p57

p57 is the least-studied WAF/CIP/KIP member with sparse data in connection with NSCLC. Alterations include frequent loss of heterozygosity with a maternal allele bias, increased methylation of the *p57* promoter region leading to decreased expression of *p57* and reduced expression in tumor tissue (compared to normal lung tissue) correlating with increased proliferation [170–172]. Proteolysis via the S-phase-associated kinase protein 2 has been reported as an important mechanism of *p57* protein downregulation [170]. It has recently been stated that decreased expression of *p57* in lung cancer is associated with poor postoperative survival time and lymph node spread [173].

Cyclin-Dependent Kinases

Specific findings regarding deregulation of CDK in NSCLC are sparse. A genomic profiling study comparing 15 early-stage lung ACA with 5 nonneoplastic pulmonary tissue specimens, found (among other cell cycle-related abnormalities) increased gene expression of *CDK2*, 4, 7 and 10 [47]. Amplification of the *CDK4* gene leading to *CDK4* overexpression has been described in lung cancer, and although it is a rare event, this underlines the importance of the RB-cyclin D pathway in lung tumorigenesis [174]. Several molecules designed to inhibit CDK exist and are currently being evaluated in clinical trials [40]. Their efficacy and possible approval for commercial use have yet to be determined. First-generation CDK inhibitors (e.g. flavopiridol) have shown low activity and/or toxicity in clinical trials [175, 176]. The possibility of other key targets of such agents and failure to reach therapeutic drug concentrations has been discussed [175, 177]. It has also been suggested that such CDK inhibitors are able to improve the efficacy of other cytotoxic drugs when used in combination [177]. A synergistic benefit has also been described for a second-generation CDK inhibitor (aminothiazole SNS-032), which sensitized radioresistant lung cancer cells to ionizing radiation [175, 178].

Aurora and Polo-Like Kinases

The Aurora family proteins are highly conserved serine-threonine kinases and are important regulators of mitosis. Aurora A has a crucial role at the G2/M phase of the cell cycle in centrosome maturation and separation during the early prophase. Gene copy number, mRNA levels and protein expression have been found to be increased in many carcinomas, correlating with disease severity [179]. Aurora B, which peaks slightly later than Aurora A, is a member of the chromosomal passenger complex and is involved in histone H3 phosphorylation, chromosomal condensation, chromosomal alignment on the metaphase plate, bipolar centromere-microtubule attachments, spindle checkpoint and cytokinesis [180]. Aurora C is thought to have a similar role to Aurora B, but seems to be restricted to the testis [179]. Aurora A and B are rapidly degraded by the APC proteasomal pathway [181]. Aberrant expression and activity of Aurora kinases disturbs mitotic checkpoints, resulting in genomic instability and aneuploidy and has been implicated in tumorigenesis for various malignancies [182]. Overexpression of Aurora A (transcript and protein) has been reported in NSCLC when compared to nonmalignant lung tissue and was associated with poor tumor differentiation [183]. One study noted that the immunohistochemical staining pattern for Aurora A is of significance, since increased perimembranous staining correlated with higher tumor stage, higher proliferative activity and was an independent predictor of poor prognosis especially for SCC, in contrast to a diffuse cytoplasmic staining pattern [184]. Previous data have shown that by phosphorylating and thus inactivating p53, Aurora A can override cell cycle arrest and apoptosis induced by cisplatin and radiation [185]. The resulting possibility of Aurora A as a predictive marker for NSCLC patients remains to be determined. Upregulation of the *Aurora B* gene and protein overexpression has also been documented in NSCLC and was correlated with lymph node spread and diminished survival [186, 187].

Inhibiting Aurora A leads to cell cycle delay at the G2/M transition followed by abnormal assembly of the microtubule apparatus [188, 189]. Inhibition of Aurora B results in polyploidy by premature mitotic exit and eventually a higher tendency to undergo apoptosis [190]. Since Aurora B allows the continuation of the cell cycle instead of mitotic arrest, the possibility of combining Aurora B inhibitors with drugs that act at different phases of the cell cycle is intriguing. Of course, the order of administration would play a crucial role for this assump-

tion. Indeed, in a study treating chronic myelogenous leukemia cells first with an Aurora kinase inhibitor followed by idarubicin or cytosine-araboside, a greater loss in viability was seen compared to cells that were treated with both agents simultaneously [191]. When both Aurora kinase subtypes are blocked, a phenotype corresponding to the sole inhibition of Aurora B is the result, suggesting that Aurora B deficiency may bypass Aurora A function during mitosis [192]. Currently, a number of Aurora kinase inhibitors are being tested in preclinical and clinical settings as antitumor agents [193]. Studies examining Aurora kinase inhibitors in NSCLC patients have reported disease stabilization as the best response, but various agents are still in the developmental phases and their role for NSCLC has yet to be investigated [193, 194].

Of the four known PLKs (1–4), PLK1 is the best characterized. PLK1 activates cyclin B1/CDK1 complexes and plays a role in centrosome maturation, microtubule dynamics and the separation of sister chromatids during the anaphase [180, 195]. By activating subunits of the APC, PLK1 is also involved in the exit of cells from mitosis [180, 195]. PLK1 expression is elevated in various types of cancer [196, 197]. High PLK1 expression has been associated with poor survival in surgically resected NSCLC patients and has been suggested as a marker of high prognostic potential [196]. Therapeutic PLK1 inhibition has recently shown modest efficacy and favorable safety in relapsed stage IIIB and IV NSCLC [198].

Transforming Growth Factor Beta

Transforming growth factor beta (TGF- β) is one of the most immunosuppressive cytokines produced by tumors and tumor-associated stromal cells, and we have recently shown that its expression correlates with significantly reduced overall survival time in patients with NSCLC [199]. In addition, TGF- β plays a crucial role in the cell cycle. SMAD are the predominant transducers of extracellular signals from TGF- β to the nucleus where they are activators of transcription. The name SMAD is a fusion of two protein names; SMAD are homologs of both the *Drosophila* protein ‘mothers against decapentaplegic’ (MAD) and the *Caenorhabditis elegans* small (Sma) pathway protein. In normal cells, TGF- β signaling causes an antiproliferative response inhibiting the G1/S transition by inducing expression of p15, p21 and p27 and suppressing cyclins A, D1 and E as well as CDK2 and 4 [62, 200–205]. Furthermore, activation of antimitogenic signaling by TGF- β has

been reported to suppress cell growth by counteracting delocalization of p27 [157, 165]. TGF- β also directly suppresses *C-MYC* expression [206]. The product of the *RB* gene has also been implicated as a target of TGF- β -induced signaling, and the activity of RB and other RB-associated molecules is required for TGF- β to efficiently suppress cell growth [206, 207]. During malignant transformation of a cell, several components of the TGF- β signaling pathway become altered, resulting in TGF- β resistance of tumor cells, which is a frequent occurrence in NSCLC [208]. TGF- β -resistant cancer cells proliferate uncontrollably, and can further increase the amount of TGF- β . In this setting, TGF- β causes invasiveness of tumor cells, immunosuppression and angiogenesis by influencing various other cells, e.g. surrounding stromal cells, immune cells, endothelial and smooth-muscle cells [209]. Resistance to TGF- β -mediated growth inhibition is often a result of mutation or loss of expression of the genes involved in the TGF- β signaling pathway. Somatic mutations are rather infrequent but can occur in *TGF- β receptors* and *SMAD* in NSCLC [209, 210]. Loss of TGF- β receptor expression has been observed more commonly and was associated with aberrant promoter methylation [211]. An in vitro study showed that overexpression of the TGF- β receptor II restored TGF- β sensitivity and reduced tumor growth in lung cancer cells [212]. Data on targeting the TGF- β signaling pathways in lung cancer are sparse; however, a recent study suggests that deactivating the inhibitory downstream molecule SMAD6 may improve patient outcome [213]. Taken together, TGF- β plays a complex and often diverse role in cancer which requires further clarification, particularly with regard to possible therapeutic approaches involving TGF- β pathways.

Ki-67

Ki-67 is a general and often analyzed cell cycle-related marker. It is a DNA-binding nuclear protein that is expressed throughout the cell cycle only in proliferating cells and is therefore often used as a marker to evaluate proliferation of tumors [133]. Although Ki-67 is one of the most-studied parameters, data concerning its prognostic value in NSCLC are inconsistent [48]. In a meta-analysis, Ki-67 overexpression was found to be a poor prognostic factor for survival in patients with NSCLC [214]. Ki-67 is used routinely for assisting the grading of tumors of the central nervous system, which may also be a possibility for NSCLC, since it has been shown that an increasing

Table 1. Reported characteristics of regulators of the cell cycle in NSCLC

Parameter	Frequently reported abnormalities	Prognostic association	Predictive association	Targeted therapy
Cyclin A	overexpression			
Cyclin B1	overexpression	poor prognosis		
Cyclin D	amplification, overexpression	poor prognosis	reduced expression may predict sensitivity to TKI [72]	
Cyclin E	overexpression	poor prognosis		
CDK	gene amplification, overexpression			flavopiridol [218], R-roscovitine [219, 220], AZ703 [221]
p16	promoter hypermethylation, gene deletion, loss of expression	poor prognosis		azacitidine [102]
p21	promoter hypermethylation, loss of expression, delocalization	poor prognosis	cisplatin sensitivity	
p27	loss of expression, delocalization	poor prognosis	cisplatin sensitivity	
p53	mutation, (overexpression)	poor prognosis (weak)	cisplatin sensitivity	
p57	promoter hypermethylation, loss of heterozygosity, reduced expression	poor prognosis		
Aurora A	overexpression	poor prognosis	cisplatin and radiotherapy sensitivity	benzopyridoindoles [222] VX-680 [223] AT9283 [224] MLN8237 [225] PF03814735 [226]
Aurora B	gene amplification, overexpression	poor prognosis		AZD1152 [227] GSK1070916 [228]
Plk1	overexpression	poor prognosis		BI 2536 [198]

Ki-67 value correlates with higher (less differentiated) tumor grades [215, 216], but in contrast to growth patterns of ACA [14, 217], this has not (yet) been shown to be of prognostic significance.

Concluding Remarks

Over the past decades NSCLC has remained the number one cause for cancer-related deaths worldwide. Early disease detection is infrequent due to the late onset of symptoms and many cases comprise primarily palliative care. The TNM classification scheme is still the most reliable means for overall prognosis, but its value for each individual is debatable. Improved markers to prognosticate survival and predict response to therapy are needed. Many reports provide evidence for the importance of regulators of the cell cycle in NSCLC. Most NSCLC have de-

tectable cell cycle abnormalities, and multiple defects are commonly seen in a single tumor. The more defective the cell cycle becomes, the more severe seem to be the consequences. Prognostic power has been attributed to various cell cycle markers, especially those involved in the cyclin D-CDK4/6-RB pathway, which play a major role at the G1/S transition. An important issue is determining which genetic alterations are driver and which are rather passenger events. A clue may be provided by their frequency and time of occurrence. For example, genetic alterations of *p16* and *CCND1* are frequently found and have been described at an early stage of tumorigenesis as well as in precursor lesions, indicating their significance. CKI seem to be associated with the response to certain anticancer drugs, since the loss of CKI may sensitize tumor cells to antiproliferative agents and thus improve efficacy. An important point when analyzing cell cycle-related parameters, which is frequently performed by immunohis-

tochemical examination of protein expression, is the exact subcellular localization. Different localizations (e.g. nuclear vs. cytoplasmic) often confer converse effects and must be adequately assessed. In addition, analyzing a single marker is likely insufficient, since interactions between cell cycle regulators affect each other and should be evaluated simultaneously. The fact that cell cycle markers are not yet part of the routine workup of NSCLC is most likely due to the prognostic strength of standard parameters (e.g. the TNM classification), the variability of technical methods of assessment of such markers and

the rather poor reproducibility of their interpretation as well as the complex and vast interactions of these cell cycle-related factors. The cell cycle as a therapeutic target is also gaining significance and different promising approaches are currently under investigation, including inhibitors of CDK and Aurora kinases. In conclusion, the cell cycle offers a multitude of prognostic, predictive and therapeutic possibilities, many of which are still in the developmental phase. Progress in this field is ongoing and has the potential to improve the current scenario for NSCLC patients (table 1).

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