Review

Cell cycle alterations and lung cancer

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Summary. It is now widely accepted that human carcinogenesis is a multi-step process and phenotypic changes during cancer progression reflect the sequential accumulation of genetic alterations in cells. The recent progress of scientific research has notably increased knowledge about biological events involved in lung cancer pathogenesis and progression, thanks to the use of molecular biology and immunohistochemistry techniques. Lots of the genetic alteration found in small cells lung cancer (SCLC) and in not small cells lung cancer (NSCLC) concern the expression of cell cycle genes, actually recognized as onco-suppressor genes and the lack of equilibrium between oncogenes and oncosuppressor genes.

The present review of literature widely describes the cell cycle control, the lung cancer molecular pathogenesis, the catalog of known genetic alterations and the recent advances in global expression profiles in lung tumors, on the basis of the various hystological types too. Such data suggest the potential use of this knowledges in clinical practice both as prognostic factors and innovative therapeutic possibilities and they impose the necessity of new studies about cell cycle control and lung carcinogenesis.

Key words: Lung cancer, Cell cycle, Oncogenes, Oncosuppressors

Introduction

A multicellular organism's cells number is physiologically regulated by a delicate equilibrium among cellular proliferation, differentiation and apoptosis. Any alteration of this equilibrium could cause an uncontrolled cellular proliferation. However, cellular genoma is constantly checked to keep its integrity. In fact, possible genomic alterations have to be corrected

before DNA duplication and cellular division. An irreparable genetic cellular alteration should automatically activate an apoptosis mechanism, a programmed cellular death, to prevent oncogenesis.

In the last years, knowledge of the cell cycle's control mechanisms, differentiation and apoptosis, has highlighted the importance of different onco-suppressive factors in development and maintenance of the transformed phenotype.

Neoplasms are characterized by a rapid and uncontrolled cellular division, with increased survival because of dedifferentiation phenomena and accumulation of genetic alterations.

The scientific approach to investigation into these disorders' pathogenesis needs a suitable knowledge of cell division's control mechanisms.

Three levels (Baserga and Rubin, 1993) can be individualized in cell proliferation's control mechanism: 1) activation of growth factor receptors from their ligands; 2) signal transduction from activated nuclear receptors; and 3) activation of cell division.

Every alteration of this chain may cause loss of cell division control, independently of all the other regulative events.

Most of the identified oncogenes can be considered components of this model: growth factors, growth factor receptors, transduction signals or transcription nuclear activators (Winford-Thomas, 1991; Marshall, 1994).

Functional alterations of any component in this complex mechanism can modify the genic expression's regulation, causing mutations or modifications of the protein products with consequent effects on cell control.

The cell cycle control

In the last years the theoretical model of cellular eucariotic division has become more evolved and complicated. The Cellular cycle takes about 36 hours *in vitro*, although it can present different variations. It is schematically separated into two principal phases - S phase (DNA synthesis, about 6 hours) and M phase (mitosis, about 30 minutes) - and two growth phases -

G1 and G2 phases (respectively 12 and 6 hours). The first growth phase, called Gl phase, is checked by different growth factors, loss of which causes cellular arrest in a phase defined as G0. When the cell is correctly developed, it enters into the so-called S phase, in which the genoma is duplicated.

After DNA duplication, the G2 phase starts. During this phase, briefer than the G1 phase, a lot of regulative mechanisms ensure that genoma replication happens only once. The last phase is the M phase (mitosis), including cariocinesis and citocinesis (Cordon Cardo, 1995).

At the end of every cycle in normal cellular division, two functionally independent cells are generated (Mac Lachlan et al., 1995).

Progression through the different cell cycle phases is checked by protein complexes: cyclines and cycline-dependent kinases (Cdk) (Murray and Hunt, 1993).

Cyclines represent a family of "periodic" proteins, synthesized during defined cell cycle moments and then quickly degraded, on the other hand, cycline-dependent kinases are cell cycle regulative proteins, constitutively expressed and active only if cycline-bound (Esposito et al., 2004).

According to current models, the enzymatically active complex is constituted of a cycline (as regulatory molecule) and a Cdk (as a catalytic sub-unity). Cycline and Cdk act through phosphorylation of fundamental elements involved in the cell cycle, such as retinoblastoma gene family's components (Dowdy et al., 1993).

The cycline family currently consists of 8 main classes, identified with letters from A to H. C, D1, D2, D3 and E cyclines reach their peak of synthesis and activity during the G1 phase (Koff et al., 1991, 1992; Xiong et al., 1991; Inaba et al., 1992; Baldin et al., 1993). A, B1, B2, and F cyclines reach their maximum levels in the later cell cycle (Knoblich et al., 1990; Pagano et al., 1992; Bay et al., 1994). G and H cyclines do not have a well-defined role and time of synthesis.

The existence of two main restriction points (starting points) has been supposed in the cell cycle, situated between the half and the end, respectively, of the G1 and G2 phases (Murray, 1994).

Cycline-dependent kinases have been similarly identified (Fang and Newport, 1991; O'Connell and Nurse, 1994; Van den Heuvel and Harlow, 2005).

Cdk2 forms a cdk2/cycline stable complex that reaches its peak of expression in G1-S transition. Moreover, it is activated by A cycline in S phase, and it seems to have a key role in the passage of the first starting point, as well as in the progression of the S phase (Xiong et al., 1993; Bates et al., 1994).

Cdk4 and cdk6 form stable complexes with D1, D2 and D cyclines. These complexes present their peak of synthesis and activity during the G1 phase and they seem to control the G1 phase.

It has also been shown that D1 cyclin-cdk4 complex interacts with PCNA (proliferating cell nuclear antigen),

suggesting a possible modulating action of this complex in well-known (DNA reparation and replication) or still unknown PCNA activity (Xiong et al., 1991; Flores-Rozas et al., 1994).

Cdk1 is activated after bonding with A and B cyclines and it is considered a key factor in the carrying out of the G2 phase, in the transition G2-M (starting point) and in the carrying out of the M phase (Draetta and Beach, 1988; Draetta et al., 1989).

Cdk5 plays an unclear role in the S phase, and cdk3 has a probable role in the transition G1-S. Cdk7's role is not defined but its activation seems to be dependent on the presence of several substrata and it probably acts during the whole cell cycle.

Proto-oncogenes and oncosuppressor genes equilibrium

Cellular proliferation is normally regulated by proto-oncogenes and onco-suppressor genes, with opposite functions on cellular growth: stimulation and inhibition, respectively. Alterations that reinforce proto-oncogenes activities promote uncontrolled tumoral proliferation. On the other hand, genetic mutations inactivating onco-suppressor genes free the cells from proliferation limits and so induce an uncontrolled cellular growth, typical of neoplasms. Therefore, the final result seems to be similar.

Proto-oncogenes (Table 1) can activate neoplastic transformation in two main ways: genetic mutation inducing an altered protein with a constantly activated function (a common event for ras members family) or overexpression of a structurally normal gene after genic amplification, rearrangement or loss of transcriptional control (e.g. overexpression of myc members family). In both cases oncogene products are constantly activated and no more regulated by an "on--off" mechanism in answer to the normal physiological stimuli. Such genes are commonly defined as dominant oncogenes since a single mutation in one of the two copies of the gene can active them. In contrast, onco-suppressor genes need a mutation on both the copies of the gene to be inactivated (Rb) or to codify an anomalous protein (p53). For this reason, onco-suppressor genes are also defined recessive oncogenes.

Onco-suppressor genes

The existence of onco-suppressor genes has been shown when their deficit has been found in particular cellular type genoma. First results have been obtained

Table 1. Oncogenes frecuently involved in lung cancer pathogenesis

N-myc	L-myc	c-myc
K-ras	H-ras	N-ras
c-erbA	c-erbB-2(Her-2/neu)	c-jun
blc-2	c-jun	

from somatic cell hybridization, where tumoral cell fusion with normal cells constantly produced a growth of non-tumoral hybrids (Sager, 1985).

These experiments showed that normal cell genetic information is able to suppress the tumoral cells' neoplastic phenotype. Such hybrid cells have an unstable cariotype and they could frequently lose a chromosome of one or the other cell ancestor. When the chromosome coming from the normal cell is lost, hybrids often restart to express the tumoral phenotype. These observations showed that the normal chromosome contains information lacking in tumoral cells and that it is able to normalize the neoplastic cells' proliferative program. These data represent the first sign that tumoral cells lose critical information about growth regulation during their progression toward the complete and irreversible malignity.

A second sign of the onco-suppressor genes' existence was derived from human genetic studies by Knudson, who postulated (in 1971) that rare childish retinoblastoma was due to two subsequent genomic alterations (Knudson, 1971). He pointed out two different forms of illness: a familial one and a sporadic one. In the sporadic retinoblastoma, observed in children without a positive family anamnesis for this pathology, he noticed the existence of two lesions, both of them consequences of somatic mutations occurring a long time after conception. About retinoblastoma he hypothesized that a lesion derives from a parent carrier or it occurs during gametogenesis, while the other one would occasionally occur as a somatic event. Several successive genetic studies confirmed Knudson's hypothesis and they identified the gene implicated on the 3 chromosome' long arm (Godbout et al., 1983).

A third way to confirm the onco-suppressor genes' existence has been suggested by the mechanisms used to induce tumoral cells to inactivate both of the copies of an onco-suppressor gene as Rb. In fact, the first copy of the onco-suppressor genes is inactivated by a somatic or germinal mutation. The chromosomal region that brings the normal allele (wild type) can be replaced by a duplicated copy of the homologous chromosomal region, where the changed allele is situated. Elimination of the normal allele, obtained by non-chromosomal disjunction, mitotic recombination or gene conversion, happens with a frequency of about 10^{-3}-10^{-4} for cellular duplication. This mechanism is also favoured by independent spontaneous eliminations of the gene's second copy, which happens with a frequency of about 10^{-6} for cellular generation. Most tumours, when deprived of acting copies of a suppressor gene (like Rb), introduce two identical changed alleli. These passages led to homozygosis of mutant onco-suppressor genes. Similarly, mapping of near chromosomal regions, which showed heterozygosis before tumoral progression, evidences a parallel evolution toward the homozygosis (or loss of heterozygosis - LOH) after phenotype transformation. Besides, repeated observations of LOH in one specific anonymous chromosomal marker in

specific type tumoral cells, suggest the presence of an onco-suppressor gene nearly mapped, the loss of which is implicated in oncogenesis. Onco-suppressor genes' location and identification require a wide molecular clonage. Therefore, it is possible to use a wide range of polymorphous DNA markers to identify the onset of repeated LOH phenomena (Harbour et al., 1988).

The purpose of the studies on onco-suppressor genes is directed at understanding their function in physiological and pathological conditions, i.e. all the mechanisms able to determine an uncontrolled growth of tumoral cells causing their functional elimination. Intercellular communications seem to be the primary mechanism to assure the integrity of normal tissue architecture. In fact, normal cells take autonomously few "decisions" and use the "suggestions" received by near cells (Hensel et al., 1990).

Single cells, not considered in global tissue structure, receive from the environment two types of growth regulator signals. A first input category constitutes proliferative stimuli: cells stimulate their neighbours' growth through mitogenic signals (polypeptidic growth factors) (Sporn and Roberts, 1985).

A lot of proteins codified by cellular oncogenes constitutively activate intracellular pathways used by normal cells to answer to mitotic exogenous stimuli. Then, oncoproteins induce a cellular state similar to the pathway after exposure of normal cells to growth factors. In this way cells purchase autonomy of proliferation, independent from surrounding stimuli.

Cells are also able to inhibit adjacent cells' growth. Onco-suppressor genes are a component of the intercellular signal chain, which makes cells able to receive and process inhibiting signals coming from the surrounding environment. When a cell loses a critical component of this signal network, it loses the possibility to answer to certain growth-inhibiting extra-cellular stimuli, sent by the surrounding environment. Different suppressor gene products should not be considered as cytostatic factors, in fact, they act like transductors of cellular growth-inhibiting signals whether intra-cellular or extra-cellular. Independently from onco-suppressor genes' specific biological functions, this model proposes a definition of these genes: a genic element whose loss or inactivation allows a cell to develop a neoplastic phenotype due to the cellular growth's de-regulation (Weinberg, 1991).

A lot of not well characterized molecules acts as exogenous stimuli, normally able to arrest cellular proliferation. A first signal is the inhibition - by contact - shown in normal cells growing in mono-stratified cultures (Stoker, 1967).

This phenomenon suggests that inter-cellular contact, mediated by surface molecules, makes a cell able to feel the narrow cells' contact and, consequently, to arrest its growth-program. Diffusible inhibitors seem to play an important role in conducting inter-cellular anti-mitogenic signals.

Different studies testified to the importance of

communication through the gaps junction (junction-shut) as an important way for the passage of low-molecular-weight molecules.

Similar importance is gained by hormones and other macromolecular inhibitors. One of these inhibitors is TGF-ß, which presents three different forms (type 1, 2 and 3) and it is able to inhibit a large range of cell growth. A cell can respond to these different negative signals in three different ways. It can simply stop its growth during one of the cell cycle phases (frequently at the end of the G1 phase, before beginning DNA synthesis). Alternatively, the cell can be induced toward its final destiny: the definitive post-mitotic differentiation. Finally, the cell can proceed to senescence and programmed death (apoptosis).

Discovery of onco-suppressor genes acting like cell cycle regulators, such as Rb and p53, suggested a connection between the cell cycle's control and the onset of cancerogenesis. p53 (wild type) controls a very important cell cycle check-point for maintenance of the genoma's integrity (Vogelstein and Kinzler, 1992). In fact, it mediates the cell cycle's arrest in the G1 phase after sub-lethal DNA damage and it seems to be involved in apoptotic mechanisms (Kaelin et al., 1991). p53 is a nuclear protein and it possesses a binding-domain to defined DNA sequences (Greenblatt et al., 1994).

p53 has been identified at first as an onco-dominant gene mapped on 17 chromosome (17p13), considering the ability of first isolated p53 cDNA's to transform cells *in vitro*, when transfected with ras or ElA (Eliyahu et al., 1984; Jenkins et al., 1984; Ebina et al., 1994). Subsequently it was noticed that DNA used in the first experiments represented a changed form of tumoral derivation. In fact, when the non-changed form (wild type) was over-expressed in cells, it inhibited its growth, whether it was a normal or a neoplastic cell, showing the p53 onco-suppressive action (Table 2) (Mercer et al., 1990).

P53 activity is regulated by multiple mechanisms among which the most common are phosphorylative events. The most important phosphorylative sites are represented by two functional dominions: the trans-activation amino-terminal dominion and the carboxy-terminal dominion (Sang et al., 1997).

It has been shown that p53, diffusely studied in the last years, is able to interact specifically with a large number of oncoproteines (SV40 large-T-antigen, E1B, HPV E6) and cellular proteins (mdm2, cdc2, some heat-shock proteins) through their bond to p53 and p53's consequent inactivation (Sang et al., 1997).

p53 takes part in gene transcription, DNA synthesis and reparation as well as programmed cellular death (Hollstein et al., 1991; Montenarh, 1992).

It has been shown that p53 (wild type) controls a very important check-point for maintenance of the genoma integrity. It mediates the cellular cycle's arrest in phase G1, after sub-lethal DNA damage (Oliner et al., 1992; Quinlan et al., 1992). In this pathway, p53 interacts with several genes: mdm2 gene's product, the first target, binds to p53 and inactivates it, establishing an autoregulated feed-back circuit. The second target, gadd45 gene, belongs to a gene family implicated in the cellular growth's arrest (Fornace et al., 1989; Puisieux et al., 1991).

The third cellular target, the gene coding for p21 protein, develops inhibitory action on multiple cycline/cdk complexes. Moreover, it has been shown that p21 also forms a complex with PCNA, a protein with a key role in DNA reparation processes (Waga et al., 1994).

The fourth target of p53, bax gene, promotes apoptotic mechanisms and it forms a heterodimer with bcl-2 gene (Selvakumaran et al., 1994). Human p53 mutations range varies according to the tumoral type.

It has been hypothesized that this range, observed in particular neoplasms, reflects the different functional ability of different p53 mutants to modulate the p53 WT (wild type)-mediated transcription. To this purpose, p53 mutants and p53 WT have been transfected together in human cellular lines, deprived of endogenous p53, to analyze their effects on p53-dependent trans-activation. This study showed that the mutants' ability to inhibit p53 WT-mediated transactivation depends on the cellular type. In fact, in cellular lines derived from pulmonary adenocarcinoma and mesothelioma, p53 WT-dependent transactivation function was strongly inhibited by all the examined mutants. Nevertheless, in cellular lines derived from prostatic cancer and osteosarcoma, the examined mutants had only minimal inhibiting effects. In cellular lines derived from hepatocellular and ovarian carcinoma, two of the examined mutants increased p53 WT-mediated transactivation, while other mutants inhibited it (Lee et al., 1987, 1995).

By consequence, p53 negative effects are due to the cellular substratum and also to particular mutants having

Table 2. Correlation between survival and p53 - pRb status in 68 lung cancer patients.

	NO PATIENTS	SURVIVAL Median (months)	P	% SURVIVAL (Follow-up: 4 years)	P
p53+	29	16	P<0.01	12	P<0.001
p53-	39	34		36	
pRb+	58	25	P= n.s.	32	P= n.s.
pRb-	10	21		28	

greater inhibitory abilities than others. This could confer a selective advantage to the transformed cell during cancerogenesis (Fujino et al., 1995; Forrester et al., 1995).

Retinoblastoma gene family

Retinoblastoma gene (RB)

Retinoblastoma gene (Rb) codes a nuclear phosphoprotein (about 105 kd weight) (Friend et al., 1986), with constant levels during a cell cycle. Nevertheless, Rb activity is regulated by cell cycle-dependent phosphorilation processes occurring periodically thanks to several cycline-kinases complexes (De Caprio et al., 1989). Therefore, pRb is hypophosphorilated in the G1 phase and it becomes hyperphosphorilated in the S phase, keeping such form during the whole G2 phase. pRb hypophosphorilated form is probably functionally active in the G0 phase and in the G1 phase's first half (Mittnacht and Weimberg, 1991).

pRb doesn't seem to possess specific DNA binding sequences and its hypophosphorilated form probably expounds its negative regulation on cell cycle forming protein complexes able to bind DNA (Ewen et al., 1993).

A segment of more than 350 amino-acids has been identified with an internal functional receptor called "pocket-domain", through which pRb can form stable complexes with cellular proteins, e.g. E2F family members, acting as transcriptional regulator factors (Chittenden et al., 1991). In fact, E2F family members could stimulate some genes' transcription.

E2F binding sites are present in genes implicated in thecS phase's induction (myc, myb, DHPR and DNA polimerase), suggesting that a pRb-E2F bond can stop the transition G1-S, preventing E2F interaction with other cell cycle stimulator genes (Johnson et al., 1993).

In 1971 Knudson proposed the so-called "two-hit hypothesis", according to which the lesion of both the alles of the same gene causes retinoblastoma's development.

Manifold studies underlined deletions on the 13 chromosome's long arm (13q14) in familiar and sporadic retinoblastoma (Knudson, 1978; Cavenee et al., 1985). Therefore, DNA sequence corresponding to this locus was cloned and called Rb1, a gene able to codify pRb, a 105-kDa protein (Fung et al., 1987; Fields and Jang, 1990). Subsequently, introduction of functioning Rb1 in cells Rb1-/-, produced the tumoral phenotype's disappearance (Huang et al., 1988; Bookstein et al., 1990). Accordingly, Rb1 was called " tumor-suppressor gene" or "anti-oncogene" or "recessive oncogene".

Following studies identified a lot of different mutations in Rb1 gene such as mutations, small or large deletions and splicing phenomena resulting in deletions of a whole exone (Horowitz et al., 1989; Kaye et al., 1990). When the Rb/p105 gene was cloned, mutations on both the Rb1 gene's alleles were found in other human tumors like osteosarcomas, prostate carcinomas, breast carcinomas, leukemias, uterine and pulmonary carcinomas. This evidence confirmed the Rb/p105 role in cellular growth control.

Rb/p105 is a common target of a lot of viral oncoproteines: E1A adenoviral proteins, polyomaviruses SV40's large T antigen and papillomavirus's E7 antigen (Moran, 1993).

It is interesting to notice that regions used by viral oncoproteines to realize their oncogenic effect, are the same used for their bond to pRb. Dominant oncoproteines, binding pRb, inhibit its function and so exert their oncogenic action. pRb binding region to viral oncoproteins, the so-called "pocket region", is constituted of two separate functional domains (Shirodkar et al., 1992; Sachse et al., 1994).

Rb/p105 activation is regulated by phosphorilative cycle dependent events. In the G0 phase and initial G1 phase, pRb appears in hypophosphorilated form.

During cell cycle progression, pRb becomes phosphorilated in at least three moments: in the middle of the G1 phase, in the S phase and during the transition G2-M. At the end of the M phase, pRb is de-phosphorilated (De Caprio et al., 1988; Goodrich et al., 1991).

PRb phosphorilation is mainly due to cyclin/cdk complexes, many of which act on pRb in vivo using a region similar to that used by viral oncoproteines (Ewen et al., 1991; Higashiyama et al., 1994).

pRb seems to determine suppressive effects on cellular growth modulating different transcriptional factors. In fact pRb hypophosphorilated form binds E2F during the G0 or G1 phase.

An pRb bond with a viral oncoprotein releases E2F which possesses "binding sites" for a lot of cellular growth stimulating genes (c-myc, c-myb, A cycline, D1 cycline etc…). This model is confirmed by the discovery that the pRb-E2F binding site is the same used by viral oncoproteines to interact with this protein (Friend et al., 1987).

Other members of the retinoblastoma gene family: P107, RB2/P130

Another two proteins, p107 and Rb2/p130, identified thanks to their interaction with the recently cloned E1A viral oncoprotein, have been shown to be structurally and functionally correlated to PRb/p105. Their structure contains a functional region (Pocket domain) presenting a greater homology with pRb/p105. Both pRb2/p130 and p107 as pRb, possess suppressive action on cellular growth (Mayol et al., 1993; Zhu et al., 1993; Claudio et al., 1994).

These proteins control the cell cycle using not entirely known ways, but a mechanism similar to that used by pRb is probably involved, through the formation of complexes with several cell cycle elements and with transcriptional factors E2F family members. In fact, Rb2/p130 and p107 show a phosphorilative cell cycle

dependent pattern, as happens for pRb (Beijersbergen et al., 1993; Baldi et al., 1996, 1997), but with a different temporal sequence in the complexes' formation (Vairo et al., 1995). Moreover, p107 and Rb2/p130 have oncosuppressive action and other functions similar to pRb ones. In fact, they are also "the target" of some onco-viruses (SV40 LTag, HPV E7 and E1A adenovirus) and they selectively interact with transcriptional factors E2F family members (Gerardts et al., 1995).

Functional relationships p53-pRb

A relationship between p53 and pRb functions in cell cycle regulation is suggested by the functions of some p53-regulated genes: MDM2 and p21. MDM2 is able to bind p53, exerting a negative control, through inhibition of its transcriptional activity and creation of a self-limiting feedback circuit (Mori et al., 1990). Moreover, MDM2 presents a Rb binding site and the interaction of MDM2 product with Rb is able to limit its functions altering the Pocket region conformation (Xiao et al., 1995). As a consequence, high expression of MDM2 could inactivate both p53 and Rb just like some viral oncoproteins.

A further interactive pattern p53/pRb seems to be favored by the p21 gene. This gene belongs to a new cell cycle regulators' family able to inhibit cycline-dependent kinases, important for Rb phosphorilation (Harper et al., 1993; Fontanini et al., 1994). Since p21 transcription is regulated by p53, it develops a clear bridge-function between p53 ed Rb activities (Durfee et al., 1993). Therefore, it has been suggested that p53 and Rb play a central role in fundamental events like cellular growth arrest and apoptosis, in answer to DNA damage, showing a strong correlation between these mutations and patient survival (prognostic role) (Esposito et al., 2005).

Other cell cycle negative regulators: inhibitors of cycline dependent cycline-kinases complexes

In these last years a new family of cell cycle negative regulators has been identified. This family's members share inhibiting functions on cyclin/cdks complex, so that they have been defined inhibitors of cycline dependent cyclin/kinases complexes (CKI). Proteins codified by these genes practice their inhibiting functions on the cell cycle through the formation of stable complexes inactivating cyclin/cdks catalytic unities.

On the base of their sequence homologies, CKIs can respectively be divided into two subgroups: the first one includes p21 and p27 and the second one comprises p15, p16 and p18.

CKIs represent the first evidence of proteins able to arrest the cell cycle progression through the interaction with other proteins that directly influence the cellular division. For this reason, CKIs are commonly considered potential onco-suppressor genes.

p21 (also known as WAF1, Cip 1, CAP 20, Sdil and mda6) was the first inhibitor of cycline-kinases complex to be discovered. Recent studies have identified specific and essential regions to inhibit cyclin-kinases complex activities. In fact, amino-acids included between 49 and 71 are essential for interaction with cdk2 and consequent inhibition of its kinasic activity. Besides, carboxy-terminal residues from 60 to 76 are essential to inhibit p21-dependent DNA synthesis in vitro. In fact, p21 is able to inactivate cycline dependent cyclin-kinase complexes: E cycline /cdk2, A cycline /cdk2, D1, D2, D3 cyclines/cdk4.

p21 transcript is directly activated by p53 and it seems to be a fundamental partner for p53. These results suggested the existence of two different p21-induction pathways, respectively p53-dependent and p53-independent. Therefore, p21 is one of the most important mediators of the p53 pathway. In fact, the p21 promoter contains two recognizable elements by p53. The p53 bond with these regions up-regulates p21 transcript. Besides, p21 is able to inhibit cdk4/D cyclin and cdk2/E cyclin complexes' kynase activities, which are absolutely necessary for S phase beginning. In the case of DNA damage, during the replication process, p21 is able to inhibit cdk2/A cyclin complex, a necessary event for cell cycle progression through the S phase (Zhang et al., 1993).

p21 can also stop DNA replication binding PCNA and forming a quaternary complex with cdk2 and A cycline. Some studies have recently proved that this interaction does not interfere with the characteristic of PCNA to participate in DNA reparation (Li et al., 1994).

p27 is a cell cycle negative regulator implicated in G1 phase arrest, which is mediated by TGF-b, intercellular contact, rapamicin and all the agents able to raise cAMP (Esposito et al., 1997).

p27 shares homology sequence regions with p21 and its product is able to stop E cycline /cdk2, A cycline /cdk2 and D cycline/cdk4 complexes' activity (Deb et al., 1994).

The recently described existence of p15 hypothesizes its potential role in TGF-ß-mediated cell cycle arrest. Such protein shares homology sequence regions with p16 and possesses the ability to form stable and inactivating binary complexes with Cdk4 and Cdk6 (Dosaka-Akita et al., 1994).

p16, another CKIs family member, specifically forms binary complexes with Cdk4 and Cdk6. p16 gene's product (also known as Ink4 or MTS1) was identified for the first time in cellular lines as a 16 kDas cdk4 subunit. Moreover, this protein is also able to inhibit cdk6 activity. p16 seems to stop cdk4 and cdk6 activation through a competitive mechanism for cycline D "binding site" and p16 inhibitory action seems to be specific for D cycline dependent kinases acting in the G1 phase (Serrano et al., 1993). In fact, p16 is only present during the final part of the G1 phase and in the S phase. Since Rb is the well-characterized G1-cdks' substratum, the existence of a feedback mechanism was

hypothesized between these two cell cycle negative regulators (Parry et al., 1995; Livingstone, 1991). This theory is supported by the discovery of p16 overexpression in Rb-/- cells, which alters the relationships between D cyclines, cdk4 and cdk6.

p18 sequence has been recently found and it presents an elevated homology with p16. It also inhibits D cyclin/Cd k4 and Cdk6 complexes' action binding Cdk4 and Cdk6.

Table 3 summarizes the gene's described functions.

Lung cancer molecular pathogenesis

Pulmonary neoplasms are one of the most important causes of death in the western world. During the 1986-1998 period, mortality for lung cancer was 75 cases/year for men and more than 25 cases/year in women in developed countries. In the same period, incidence of lung cancer overcame the 40 new cases per year for women and 80 new cases per year for men in the United States.

During the 1973-1991 period, female incidence doubled in United States, while a trend of reduction is noticed for Caucasian men in the last years. The curve related that African men had a discontinuous course in that period of time.

Lung cancer turns out to be the first cause of death for tumor in men and in Caucasian women, while it is the second cause, after breast cancer, for African women. This datum, compared with an increased mortality in the 1950-1991 period, points out that this tumor is often diagnosed in a advanced state, with few possibilities of cure.

In Italy, mortality for lung cancer is 28.000 cases per year with percentage values of 28.4% in men and 6.9% in women (La Vecchia and Levi, 2003).

There are a lot of risk factors currently considered for lung cancer, such as smoking cigarettes, exposure to polycyclic aromatic hydrocarbons, radon and asbestos exposure.

Although exposure to these factors can be checked against changing life style and environment, lung cancer's prognosis is often fatal. The main reason is the difficult precocious diagnosis of this type of neoplasia, because it generally clinically reveals itself only in advanced state. In fact, despite the effected different therapeutic interventions up to now, also in combined form, progress in this field did not have a real effect on patient survival (Yokota et al., 1987; Shottenfeld, 1996).

The recent progress of scientific research has notably increased knowledge about biological events involved in lung cancer pathogenesis and progression. A great deal of genetic alterations were found both in small cell lung cancer (SCLC) and in non small cell lung cancer (NSCLC). Lots of them concern the expression of cell cycle genes, actually recognized as onco-suppressor genes.

A large number of these genes have not yet been identified and characterized. Between well-known genes, retinoblastoma (Rb) and p53 genes exhibit a fundamental role in lung cancer's development (Suzuki et al., 1992).

We are going to describe p53 mutations spectrum in lung cancer, such mutations' origin and the hypothesis

Table 3. Cell cycle regulators.

PROTEIN	SPECIFIC FUNCTION	PHASE OF ACTION
A cyclin	Cdk2 activation	Transition S-M
B1 cyclin	Cdc2 activation	Progression in M
B2 cyclin	Cdk4 and cdk6 activation	
C cyclin		
D1 cyclin		
D2 cyclin	Cdk2 activation	
D3 cyclin	Cdk2 activation?	
E cyclin		Progression in S
F cyclin		During G2
G cyclin	?	Ubiquitarian?
H cyclin	Cdk7 activation	Ubiquitarian
cdc2	H1 hystone phosphorylation	Progression in M
cdk2	DNA reparation	Through S
cdc3	?	Through G1
cdk4	pRb phosphorylation	During G1
cdk5	Tau and cerebral neurofilament proteins phosphorylation	During G0
cdk6	pRb phosphorylation	Through G1
cdk7	Cdks activation and RNA polimerase II CTD phosphorylation	Ubiquitarian
p21	Cdks 2, 4, 6 and PCNA-mediated DNA replication inhibition	During G1 and S
p27	Cdk2 inhibition after intercellular contact or pretreatment with TGF-b	During G1
p15	Cdks 4 and 6 inhibition after treatment with TGF-b	During G1
p16	Cdks 4 and 6 inhibition	During G1

produced by p53 alterations analyzed in precancerous or neoplastic lesions.

p53 mutations represent extremely common events in lung cancer. The frequency of these alterations changes according to the different histologic types. They occur in a percentage that varies from 57% to 80% in SCLC and from 36% to 60% in NSCLC. Between NSCLC, squamous carcinomas and great cells carcinoma present a higher frequency of p53 mutation than adenocarcinomas (Fontanini et al., 1992, 1993; Reissman et al., 1993; Minna, 1993; Fontanini et al., 1993).

Such mutations happen in the whole p53 sequence and, apart from less frequent deletions and insertion phenomena, they are essentially different types of transversion. The spectrum of such mutations' frequency changes according to the histologic type, as well as in relationship to the exposure to some risk factors and particularly to cigarette smoke.

Cigarette smoke is a complex mixture with thousand of constituents, like cytotoxic substances (formaldehyde, acetaldeide etc…), toxic agents (carbon monoxide, nitric oxide etc…) and carcinogenic agents (2-nitropropanos, N-nitrosamine, benzopirene etc…). These last showed carcinogenic activity *in vitro* (Shottenfeld, 1996). Nevertheless, the real contribution of many of these agents to lung carcinogenesis has not been defined yet. Cigarette smoke is considered to be responsible for 90% of male lung cancers and 78% of female lung cancers. Benzopirene, one of the most frequent carcinogenic agents in tobacco smoke, selectively induces G:C-T:A transversion in vitro, an extremely common p53 alteration in human lung carcinoma. It has been recently shown that benzopirene is able to specifically interact with p53, altering its structure.

Frequency of p53 mutations and G:C-T:A transversion in not transcribed DNA has meaningfully been correlated to cigarette consumption. Besides, cigarette smoke and some of its components have been shown to increase mutation frequency *in vitro* and to damage DNA reparation. p53 has a key role in this last process (Esposito et al., 1997).

Research conducted on the association between altered p53 expression in lung cancer and cigarette consumption in these patients have shown a real correlation between p53 overexpression and cigarette smoke. This correlation was stronger in patients with daily cigarette consumption, whereas non-smoker patients affected by lung cancer had a lower frequency of p53 mutations (Downes and Wilkins, 1994). These acquisitions suggest p53 alterations' important role in tobacco smoke carcinogenesis and they push the investigations on p53 alterations into a new category of individuals at risk: "passive smokers".

The approach to the analysis of p53 alterations includes molecular biology and immunohistochemistry techniques.

The study of DNA sequences is essential for a precise identification of p53 mutations. Non-altered form of p53 protein possesses a too brief half-life to make it identifiable by immunohistochemistry method. On the other hand, the search for p53 altered forms, possessing an increased halflife, can be easily conducted with classical immunocytochemistry method, which is able to identify its accumulation (Iggo et al., 1990).

The immunocytochemistry localization of p53 modified form is essentially nuclear, with variable intensity of its activity, comparable to the quantity of accumulation.

In order to investigate p53 alterations' chronology in lung cancer, many searches have been conducted about p53 expression in precancerous lesions, neoplasms and metastatic lesions (table number 4). Immunohistochemistry has not identified p53 alterations in normal epithelias, squamous hyperplasias and metaplasias, while studies of dysplastic lesions and in situ carcinomas have underlined a frequent p53 over-expression (from 50% to 75% in different case histories). This parameter increases according to the dysplasia's severity. Nevertheless, p53 stabilization found in these cases, could refer to cellular pattern alterations of p53 turnover regulation, rather than to real punctiform mutations. p53 over-expression, associated with neoplastic lesion genesis and progression, could be considered just like a beginning and functional alteration (Minna et al., 1991).

Where proliferative *in situ* lesions and invasive neoplasms coexisted, p53 has been shown to be expressed in both the lesions or it was not found in either of them. Finally, studies have been conducted on primitive neoplasms and lymphonodal metastasis to determine if altered p53 expression could be acquired or maintained in metastatic lesions, if found in primitive tumors. Different authors have described high p53 levels in metastasis from p53 negative neoplasms (De Marini, 1993).

However, even if p53 prognostic value in lung cancer is still controversial, proliferative frequency is universally considered a negative prognostic factor in manifold neoplasms (Fontanini et al., 1992; Groeger et al., 2004).

The attention of researchers is focused on those cell cycle markers presenting an immunohistochemistry expression correlated to p53 expression. Between these markers, proliferating cell nuclear antigen (PCNA) has

Table 4. Potential sequence of p53 genetic events in lung carcinogenesis.

TISSUE	P53	
	HHC	MOLECULAR
Normal mucousa	Negative	Negative
Metaplasia	Negative	Negative
Dysplasia	Positive	Changed
In situ carcinoma	Positive	Changed
Invasive carcinoma	Positive	Changed

been shown to be the most useful. PCNA expression pattern has been well characterized during the cell cycle and it correlated with DNA replication and cellular proliferation (Fontanini et al., 1992).

PCNA immunohistochemistry expression has been found in the majority part of lung cancers studied (from 95% to 100% of the different case histories) and positively correlated to p53 overexpression (Esposito et al., 1996). These data find support in some recent observations. Mercer and colleagues have shown that p53 non-altered form (wild type) negatively regulates PCNA expression both during mRNA synthesis and protein expression, while p53 altered form seems to be able to directly promote PCNA expression (Deb et al., 1994).

We have to remember that PCNA is commonly considered a negative prognostic indicator in different neoplasms (Ramael et al., 1994). Evaluation of this data demonstrates p53 alterations' important role in lung cancer progression. Data of the first experiments about p53 "gene transfer", through injection of retroviral vectors in lung cancer patients, have been recently produced (Roth et al., 1996). This study, conducted on a total of nine patients, showed a local regression of disease in three patients and a stabilization of the disease in another three. Increased apoptosis was found in all the biopsies effected after the treatment. Such data are promising for the gene therapy of lung cancer, which is the future of therapy.

Rb/p105 inactivation is a common event in lung cancer, more frequent in SCLC than in NSCLC. In SCLCs, Rb alterations can be found in a high percentage of cases, from 88% to 100% of the bioptic samples and from 33% to 100% in cellular lines from different records of cases (Baldi et al., 1996). In NSCLCs, Rb alteration has a much lower incidence, with percentages from 1% to 56% in the different records of cases (Suzuki et al., 1992; Baldi et al., 1996; Esposito et al., 1996) and they are more frequent in squamous carcinomas than in adenocarcinomas.

The different percentages of pRb/p105 alterations reported in the literature also depend on the different approaches and techniques used. In fact, immuno-histochemistry is able to identify genic inactivation in a greater number of cases than Northern blot or Southern blot analysis. There are different explanations. The first one is immunohistochemistry's high sensitivity, due to the possibility of finding gene expression products in every single cell of the sample. Besides, pulmonary carcinoma samples frequently contain a lot of non-malignant elements such as fibroblasts, vascular elements and inflammatory cells. When such samples are examined by immunohistochemistry, not-malignant cells present an Rb normal expressive pattern, which is often absent in neoplastic elements. On the contrary, in these types of neoplasms it is possible to find a normal transcript in Northern blot analysis and a normal banding in Southern Blot analysis, both referring to the nucleic acids coming from not transformed cells. Besides, even

if immunohistochemistry does not allow qualification of the type of mutation, it possesses an elevated sensitivity, which points out genic inactivation phenomena, in samples with an unclear neoplastic component too. This phenomenon has been observed in small-cell-lung-cancer cell lines carefully studied. Rb transcripts appeared normal in Northern blot analysis but they showed small deletions and mutations if analyzed with techniques such as RNAase protection and RT-PCR, and they were associated with the absence of nomal pRb/p protein expression (Yokota et al., 1987; Fontanini et al., 1992). Additionally, an alteration of the pRb expression pattern has been described in 10 of 36 NSCLCs, all of them except one presenting a normal transcript (Xu et al., 1991). In this situation, use of bioptic samples instead of cellular lines avoids possible adulterated results caused by in vitro culture.

Xu and colleagues have shown that the absence of pRb expression in NSCLCs is correlated to the pathological tumoral stage, since such protein's expression decreases in advanced tumors (Xu et al., 1991). Moreover, Higashi and colleagues highlight that pRb expression is meaningfully greater in tumors with a low grade of differentiation than in well-differentiated tumors (Kitagawa et al., 1994).

Concerning pRb alterations' prognostic, Xu and colleagues attribute a negative prognostic meaning to p105 alteration in NSCLC (Xu et al., 1991).

It is diffused opinion that alterations in cell division regulating mechanisms, caused by pRb functions' inhibition, are counterbalanced by p53 functions that, in some circumstances, induces programmed cellular death (apoptosis). Therefore, loss of functions of an onco-suppressor gene would be offset by the activation of the other one, in a sort of emergency mechanism that should intervene to avoid an uncontrolled proliferation.

Xu and colleagues, hypothesizing an independent prognostic role for Rb in the NSCLC, also suggest the eventuality of a worse prognosis for those neoplasms with both Rb and p53 inactivation (Xu et al., 1991). However, we did not confirm these results in a recent immunohistochemistry study in which we evaluated pRb and p53 "status" in 68 samples of NSCLC (Esposito et al., 1996). In fact, statistical analysis of immuno-histochemical data did not underline any correlation between the lack of pRb expression and patient prognosis, even when pRb deficit was correlated to p53 altered expression. On the contrary, p53 confirmed its negative prognostic value.

In conclusion, although p53 and pRb check together an important restriction point in the cell cycle, it doesn't seem that their contemporary inactivation can get worse lung cancer patients' prognosis. Nevertheless, the importance of these two oncosuppressor genes' role in lung cancer pathogenesis clearly emerges from these study.

Further research is necessary to understand the real possibilities of survival in lung cancer patient with pRb/p105 inactivation, as well as to differentiate such

possibilities within the different histological types. These studies require the standardization of used methods and the collection of long-term follow-up inactivation data. Interpretation of these data must consider other alterations found in the other cellular growth regulating genes, to shape the genetic alteration pattern of each neoplasia and their prognostic value, essential information to individualize patients that really could benefit from surgical therapy.

In a recent study, immunocytochemistry expression has been investigated in 77 lung carcinomas of different histotypes. The three genes of the Rb family (Rb/p105, p107 Rb2/p130) act differently in the various histological types, probably according to the different qualitative and quantitative involvement of the three genes in lung cancer pathogenesis.

Neuroendocrinal tumors (carcinoids and mycrocitomas) have shown lower expression levels of the three proteins in comparison with the different forms of epithelial tumors (epidermoidal carcinomas, adenocarcinomas and bronchiolo-alveolar carcinomas). The absence of pRb2/p130 expression has been statistically correlated to the low grade of differentiation in all the investigated tumoral histotypes. Furthermore, pRb2/pl30 expression has been shown to be correlated - in negative way - to PCNA, a well- characterized proliferation index, within the histological types traditionally considered aggressive, such as mycrocitoma and adenocarcinoma. Such correlation, absent in epidermoidal carcinomas, was interestingly positive in carcinoids. These data have underlined the important Rb2/pl30 role in SCLCs, suggesting a different involvement of such proteins according to the different origins of neoplasia (Gerardts et al., 1995). Besides, Rb2/pl30 and pRb have never been found simultaneously absent in the same sample of NSCLC or cellular line. Nevertheless, these studies included quite a small number of cellular lines and pulmonary carcinomas. As a consequence, these data need to be confirmed on a larger scale, looking for some statistically significant correlations between the alterations of p16 and pRb expression and the impact of such alterations on lung cancer patients survival.

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