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Genetic alterations of multiple tumor suppressors and oncogenes in the carcinogenesis and progression of lung cancer

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Lung cancer has become the leading cause of cancer death in many economically well-developed countries. Recent molecular biological studies have revealed that overt lung cancers frequently develop through sequential morphological steps, with the accumulation of multiple genetic and epigenetic alterations affecting both tumor suppressor genes and dominant oncogenes. Cell cycle progression needs to be properly regulated, while cells have built-in complex and minute mechanisms such as cell cycle checkpoints to maintain genomic integrity. Genes in the p16INK4A-RB and p14ARF-p53 pathways appear to be a major target for genetic alterations involved in the pathogenesis of lung cancer. Several oncogenes are also known to be altered in lung cancer, leading to the stimulation of autocrine/paracrine loops and activation of multiple signaling pathways. It is widely acknowledged that carcinogens in cigarette smoke are deeply involved in these multiple genetic alterations, mainly through the formation of DNA adducts. A current understanding of the molecular mechanisms of lung cancer pathogenesis and progression is presented in relation to cigarette smoking, an absolute major risk factor for lung cancer development, by reviewing genetic alterations of various tumor suppressor genes and oncogenes thus far identified in lung cancer, with brief summaries of their functions and regulation.
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Keywords: tumor suppressor; oncogene; lung cancer

Introduction

Lung cancer has become the leading cause of cancer death in many industrialized countries claiming more than 160 000 and 50 000 lives annually in the United States and Japan, respectively (Minna *et al*., 1997; Statistics and Information Department, Minister's Secretariat, 1999). A better understanding of the molecular pathogenesis of this fatal disease is thus urgently needed in order to achieve a preventive or therapeutic breakthrough aiming at a reduction in the number of deaths. Multiple morphological steps have been recognized in the carcinogenic process of lung cancer. In the development of squamous carcinoma at the bronchial epithelium, oncogenic triggering converts normal bronchial epithelium into hyperplastic, metaplastic and dysplastic lesions. After these premalignant stages, lung cancer develops as a carcinoma *in situ* (CIS), and then emerges as an overt squamous cell carcinoma. Adenocarcinoma is also considered to develop at least in part from premalignant precursor lesions such as atypical adenomatous hyperplasia (AAH). Molecular biological studies have demonstrated that overt cancers carry multiple genetic and epigenetic alterations, indicating inactivation of tumor suppressor genes and activation of dominant oncogenes during the processes of carcinogenesis and subsequent progression of lung cancer. In fact, accumulating evidence points to the multi-step accumulation of genetic and epigenetic changes, which often accompany the sequential morphological changes (Figure 1).

It is widely acknowledged that cigarette smoking is a dominant risk factor for lung cancer, and that carcinogens in the smoke are main initiators of lung cancer by inducing multiple genetic alterations, mainly through the formation of DNA adducts. Fingerprints of such genetic insults can be seen, for example, in the mutational spectra of *p53* and *KRAS*. Proper cell cycle regulation and checkpoints are crucial for maintaining genomic integrity, and their abrogations are thought to contribute to genomic instability (Dhar *et al*., 2000), thereby playing an important role even in the early steps of cancer development. Many of the tumor suppressor genes and oncogenes altered in lung cancer are known to play a role in the regulation of cell cycle progression in either a direct or an indirect manner, and a considerable proportion of the lung cancer-related genes are a component of the checkpoint mechanisms. In addition to the genetic and epigenetic changes to genomic DNA, the presence of genetic instability in lung cancer is also reflected by frequent structural and numerical changes of chromosomes (Testa, 1996; Haruki *et al*., 2001). In this study, we will mainly focus on the genetic changes, in relation to cigarette smoking, and their roles in the pathogenesis and progression of lung cancer.

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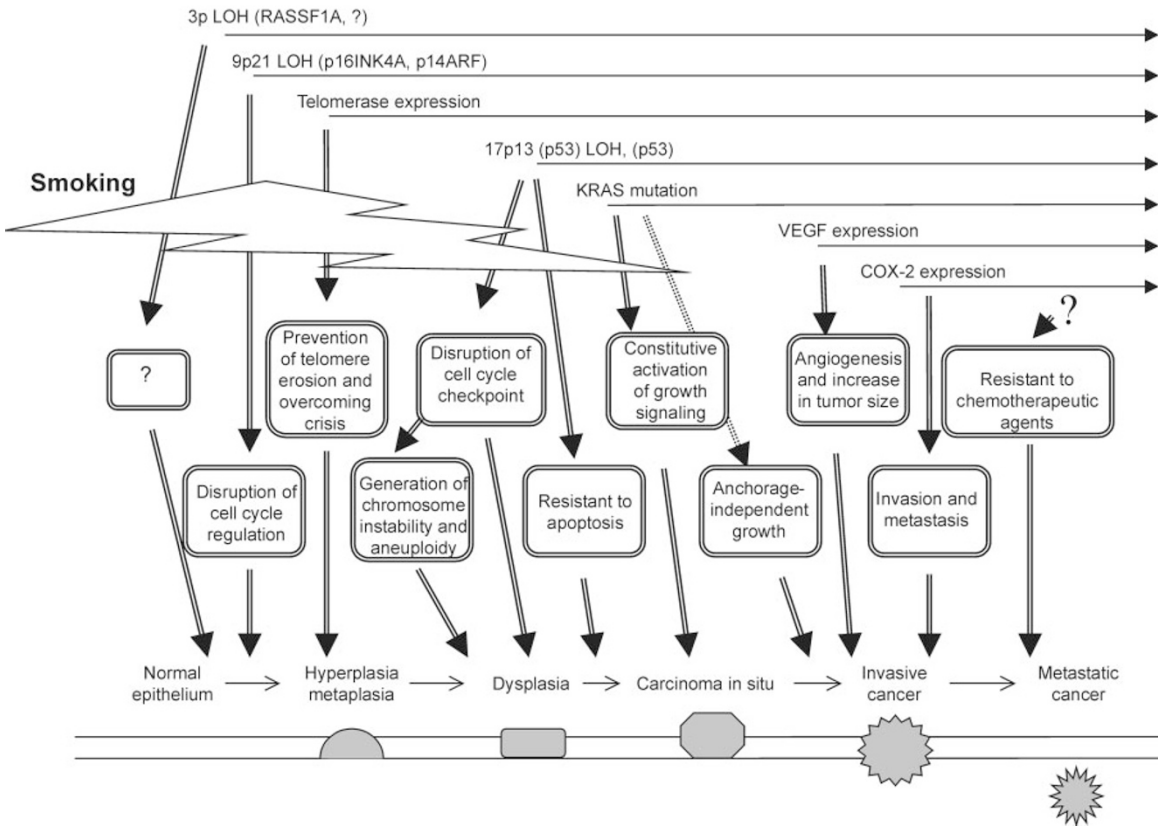

Figure 1 Accumulation of alterations in the multi-step progression of lung carcinogenesis. Oncogenic triggering events, most significantly due to exposure to carcinogens in cigarette smoke, result in the accumulation of genetic and epigenetic alterations, which convert normal bronchial epithelium into hyperplastic, metaplastic and dysplastic lesions, and then carcinoma *in situ* (CIS) and overt squamous cell carcinomas. Adenocarcinoma is also thought to develop similarly in a multi-step fashion at least in part from pre-malignant lesions in the peripheral airways such as atypical adenomatous hyperplasia (AAH). Supposed biological consequences resulting from the genetic and epigenetic alterations are indicated

Tumor suppressors and growth inhibitory signals

During the processes of lung carcinogenesis and progression, multiple tumor-suppressor genes are inactivated. Alterations inactivating tumor suppressor genes usually involve two events: deletion of a large chromosomal DNA segment of one allele and a smaller mutational or epigenetic event in the other allele. Previous studies on loss of heterozygosity (LOH) have provided clues to the localization of tumor suppressor genes involved in the pathogenesis. Deletions of 3p, 9p21, 13q14, and 17p13 are frequently observed even in the early lesions preceding the conversion into overt lung cancer. Allelic loss of 3p is among the most frequent and earliest events in lung cancer development, suggesting that an important tumor suppressor gene(s) may reside in this region. The 9p21, 13q14 and 17p13 regions harbor the *p16INK4A/p14ARF/p15INK4B*, *Retinoblastoma* (*RB*) and *p53* genes, inactivation of which has been shown to play a fundamentally important role in the pathogenesis of lung cancer. It is of note that these tumor suppressor proteins compose functional linkages, i.e., p14ARF-p53 and p16INK4A-RB pathways, and that they are components of checkpoints and growth inhibitory pathways as discussed below (Figure 2).

p53/MDM2/p14ARF pathway (p53, 17p13.1; MDM2, 12q14.3-q15; p14ARF, 9p21)

The *p53* tumor suppressor gene is now regarded as a guardian of the genome, playing a central role in cell cycle checkpoints. Upon damage of DNA in the genome, the ATM and ATR kinases are activated, resulting in the phosphorylation and consequential activation of the p53 protein directly at Ser 15 and indirectly via CHK2 or CHK1 at Ser 20 (Figure 3). The activated p53 transactivates its target genes including *p21CIP/WAF1*, *14-3-3σ*, *BAX*, and *GADD45* (Yu *et al*., 1999). Up-regulation of *p21CIP1/WAF1* (Harper *et al*., 1993; el-Deiry *et al*., 1993) and *14-3-3σ* (Hermeking *et al*., 1997) by p53 imposes G1 and G2 arrest, respectively, while the activated p53 is also believed to participate in DNA repair through the induction of *p53R2* expression (Yamaguchi *et al*., 2001). Alternatively, unrepairable DNA damage may lead to apoptotic cell death via p53-dependent induction of the expression of various downstream

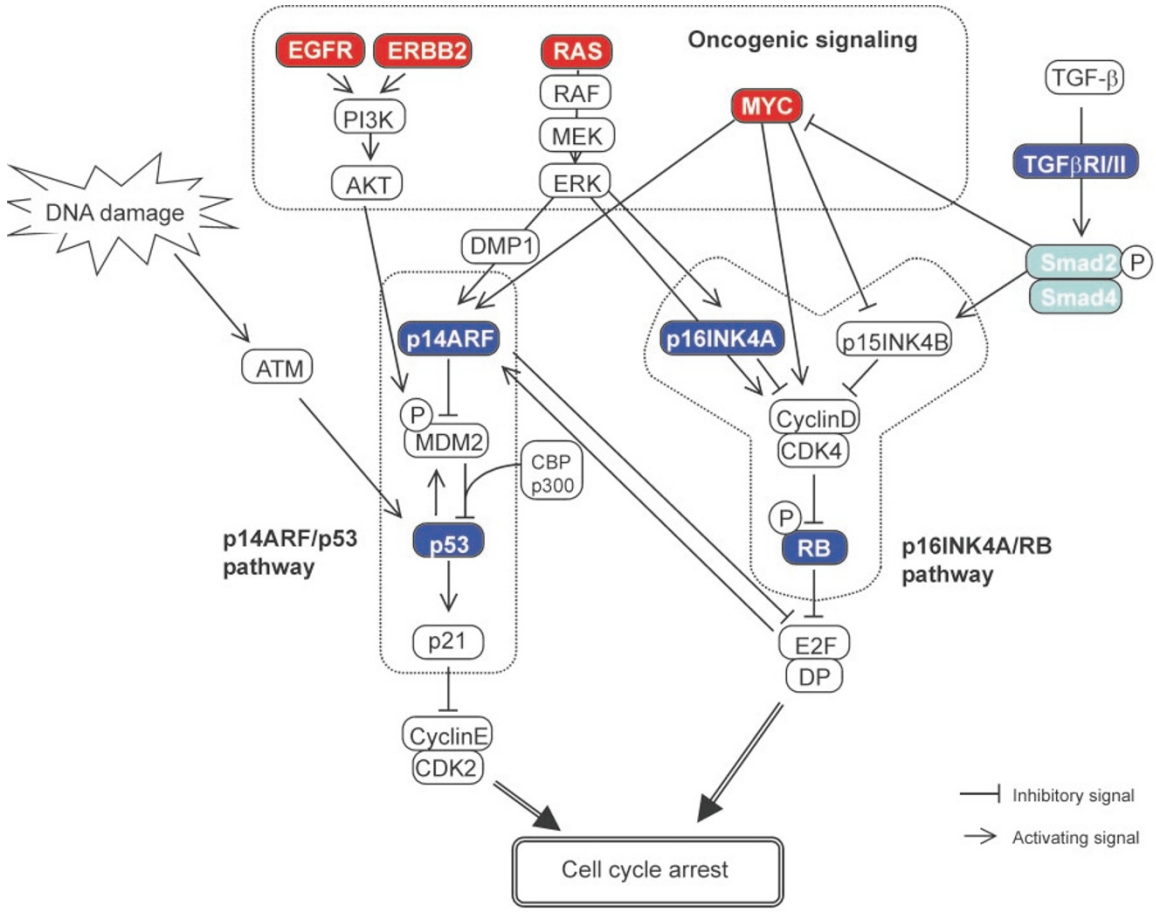

Figure 2 Alterations of tumor suppressor pathways in lung cancer. Two major pathways (i.e., p14ARF-p53 and p16INK4A-RB) involved in the pathogenesis of lung cancer are functionally linked and play roles as a component of checkpoints and growth inhibitory pathways. In addition, signals from various oncogenes and growth factors are also linked to these pathways. Note that many of the components are inactivated in lung cancer. Molecules with frequent activating alterations in lung cancers are marked in red. Molecules showing frequent and infrequent inactivations are indicated in dark and light blue, respectively

genes including the pro-apoptotic *Bcl-2* family (*BAX*, *NOXA*, and *PUMA*) (Miyashita and Reed, 1995; Oda *et al*., 2000a; Yu *et al*., 2001), *APAF-1* (Robles *et al*., 2001), *PIG3* (Venot *et al*., 1998) and *p53AIP* (Oda *et al*., 2000b). Phosphorylation at Ser 46 of the p53 protein by HIPK2 has been suggested to play a key role in the induction of apoptosis by p53 in concert with the phosphorylation of Ser15 and Ser20, described above (D'Orazi *et al*., 2002; Hofmann *et al*., 2002). The regulation of centrosomal duplication has also been linked to p53 and its effecter p21CIP1 (Tarapore *et al*., 2001).

Since the initial report on *p53* mutations in lung cancer (Takahashi *et al*., 1989), enormous numbers of *p53* mutations have been documented, providing solid evidence for the presence of mutations in ~90% of SCLC and 40~70% of NSCLC, depending on histological type as well as for clustering of *p53* mutations at hotspot codons, i.e., codons 157, 245, 248, and 273 (Harris, 1996). These hotspot codons harbor CpG sites, while 5-methyl-cytosine has been suggested to enhance the formation of DNA adducts at the guanine residue (Hainaut and Pfeifer, 2001). The resultant bulky DNA adducts, which are mainly formed by carcinogens in cigarette smoke, preferentially cause a guanine to thymine transversion. Highly sensitive densitometric examination has revealed that smoking induces *p53* mutations even in bronchial epithelial cells of normal morphological appearance (Hussain *et al*., 2001). In addition to a role for p53 inactivation at the early stages of lung cancer development, accumulating evidence suggests a possible relation to a poor prognosis after surgical removal of lung cancers (Mitsudomi *et al*., 2000).

MDM2 (HDM2), a ubiquitin E3 ligase, interacts with p53 and down-regulates p53 protein expression, thereby functioning as an oncogene (Haupt *et al*., 1997; Kubbutat *et al*., 1997), p53 in turn up-regulates MDM2 expression, which functions as an autoregulatory negative feedback loop. MDM2 is also positively regulated through its phosphorylation by AKT, a downstream effecter of growth factor signals (Zhou *et al*., 2001), Whereas amplification of MDM2 occurs in 14% of osteosarcomas (Ladanyi *et al*., 1993), MDM2 has been reported to be overexpressed without gene amplification in 25–50% of NSCLC, interestingly, in

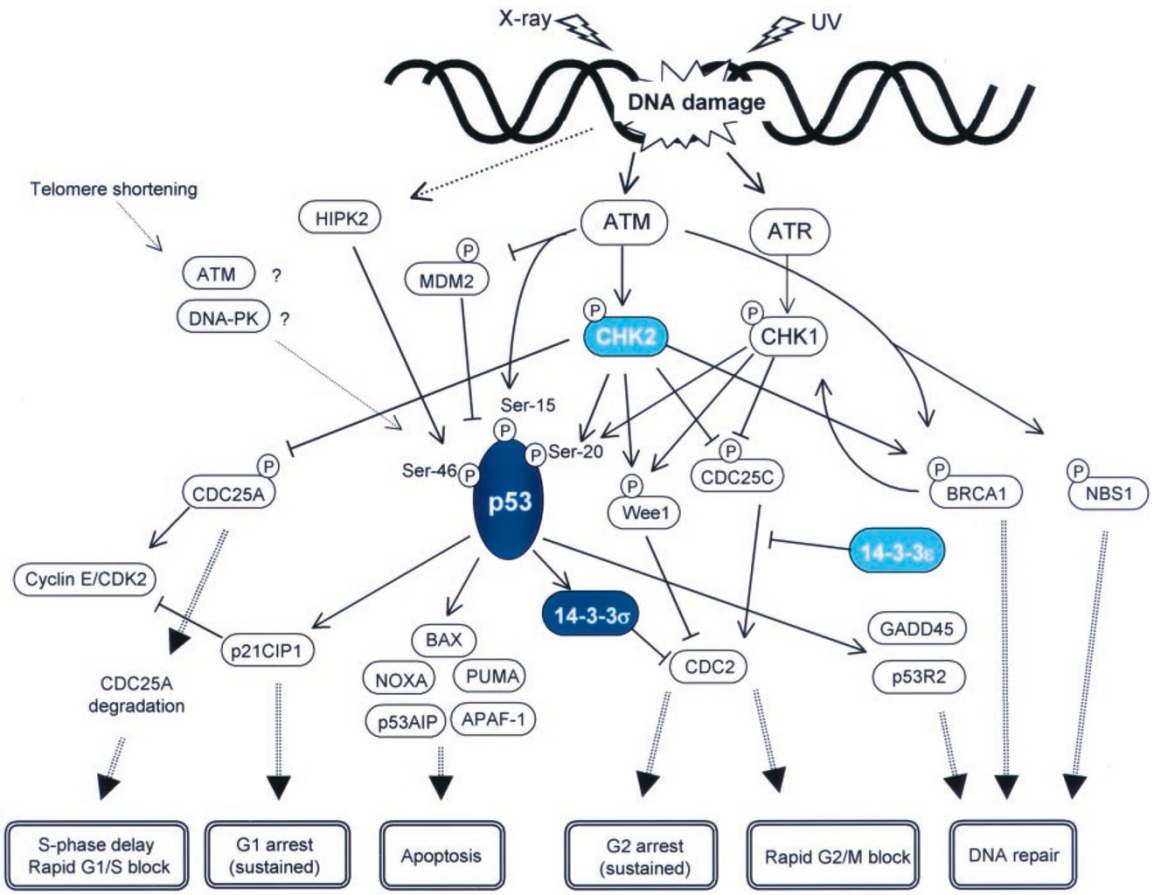

Figure 3 p53 functions and cell cycle checkpoints. The p53 tumor suppressor gene plays a central role in the cell cycle checkpoints and is frequently inactivated by genetic alterations in lung cancer. DNA damage in the genome activates the ATM and ATR kinases and results in the phosphorylation and consequential activation of the p53 protein, leading to the transactivation of various target genes of p53, which function in cell cycle arrest, apoptosis and DNA repair as well as proper centrosome duplication. Most lung cancers contain p53 mutations conceivably triggered by the formation of bulky DNA adducts of carcinogens in cigarette smoke

association with a favorable prognosis (Higashiyama *et al*., 1997; Ko *et al*., 2000). p14ARF, which is encoded by an alternative coding frame of the *p16INK4A* locus, exerts growth inhibition by inhibiting the ubiquitin E3 ligase activity of MDM2 (Honda and Yasuda, 1999) and by sequestering MDM2 into the nucleolus (Weber *et al*., 1999; Zhang and Xiong, 1999). It is of note that p14ARF is induced by oncogenic signals (MYC, RAS, and E2F1), suggesting the existence of a negative feedback mechanism (Zindy *et al*., 1998; Palmero *et al*., 1998; Bates *et al*., 1998). Deletion of p14ARF may therefore potentiate the tumor-promoting activity of these oncogenes. In this connection, although mutations specifically targeted at inactivating p14ARF instead of p16INK4A have not been found in lung cancers, loss of p14ARF expression has been reported in about 60% of lung cancers (Sanchez-Cespedes *et al*., 1999).

RB (RB1) and CDK inhibitors

The p16INK4A-RB pathway negatively regulates cell cycle progression. While RB protein is hypophosphorylated at the G1 stage, RB is sequentially phosphorylated by CyclinD–CDK4/6 and CyclinE–CDK2 during the G1/S transition. This modification leads to the dissociation of RB from E2F/DP heterodimers, leaving them in a transcriptionally active state. These heterodimers activate the expression of genes involved in cell proliferation and promote cell cycle progression as well as differentiation, development, and apoptosis (Muller *et al*., 2001; Ishida *et al*., 2001; Kalma *et al*., 2001; Ren *et al*., 2002). p16INK4A keeps the RB protein in the hypophosphorylated form by inhibiting CyclinD–CDK4/6 activity, and consequently prevents activation of the E2F/DP heterodimers.

Alterations of RB are detected in almost all SCLCs (>90%). This unusually high incidence of RB abnormalities is seen only in a very few types of human tumors such as SCLC and retinoblastoma. In SCLC tumor specimens, the *RB* locus in the 13q14 region almost invariably exhibits the loss of one allele and a structural alteration of the other allele of the *RB* gene, which is due to subtle changes including small deletions, non-sense mutations and splicing abnormal-

ities rather than large deletions. Consequently, the *RB* transcript and protein are very frequently absent or abnormal in SCLC (Harbour *et al*., 1988; Yokota *et al*., 1988; Hensel *et al*., 1990). In contrast, loss of RB protein expression is rather rare in NSCLC (15–30%) with a higher frequency in late-stage NSCLC than in early-stage tumors, suggesting a possible association with tumor progression (Xu *et al*., 1991). In addition, LOH at the *RB* locus is detectable in dysplastic lesions (Wistuba *et al*., 1997). The inactivation of RB in NSCLC does not seem to correlate with time to relapse or death (Reissmann *et al*., 1993; Shimizu *et al*., 1994), whereas a significant association of the RB status with a poor prognosis has been suggested to be present when considered in combination with the p53 status in early-stage NSCLC (Xu *et al*., 1996). Although alterations of the *RB* gene occur more frequently in squamous carcinomas, which are associated with heavier smoking, than in adenocarcinomas, no association between the RB status and smoking history was observed (Shimizu *et al*., 1994). It is interesting that RB alterations in large cell carcinomas with neuroendocrin features were detected as frequently as in SCLC, suggesting a possible relationship between genetic alterations of the *RB* gene and neuroendocrine differentiation (Cagle *et al*., 1997). This is supported by the experimental data showing that *Rb*+/− mice developed neuroendocrine tumors of various origins after long latency periods (Nikitin *et al*., 1999). Mutations in *Rb* and *p53* appear to cooperate in tumorigenesis as shown using mice deficient in the respective genes, and this may be partly because *p53* mutation may allow cells to escape from p53-dependent apoptosis induced by the inactivation of the Rb pathway (Morgenbesser *et al*., 1994; Vogelstein *et al*., 2000). Indeed, virtually all SCLC tumor specimens show both p53 and RB alterations. In this connection, it has been reported that E2F1 is frequently overexpressed in SCLC (Eymin *et al*., 2001). Details on frequent epigenetic alterations of the *p16INK4A* gene in NSCLC will be covered in another chapter dealing with epigenetic changes, but it is interesting to note that in the two major classes of lung cancers (i.e., SCLC and NSCLC), p16INK4A and RB are reciprocally inactivated, resulting in the inactivation of the same p16INK4A/RB pathway (Otterson *et al*., 1994; Kelley *et al*., 1995) and the activation of E2F/DP signaling in virtually all types of lung cancers.

p16INK4A (*CDKN2A*, 9p21), belongs to one of the two CDK inhibitor families together with p15INK4B (*CDKN2B*, 9p21), p18INK4C (*CDKN2C*,1p32), and p19INK4D (*CDKN2D*, 19p13), all of which inhibit the activity of CDK4/6. The other family consists of p21CIP1/WAF1 (*CDKN1A*, 6p21.2), p27KIP1 (*CDKN1B*, 12p13.1-p12), and p57KIP2 (*CDKN1C*, 11p15.5), which have a wider spectrum of target CDK molecules. In addition to the level of p16INK4A, the expression level of p27KIP1 has been shown to be reduced in 70% of NSCLC tumor specimens in association with a poor prognosis (Yatabe *et al*., 1998), due to the abrogation of its ubiqutin–proteosome-mediated degradation (Loda *et al*., 1997). No genetic alteration of the *p27KIP1* gene, however, has been reported thus far. Interestingly, SCLC exhibits significantly increased p27KIP1 expression (Yatabe *et al*., 1998), which may favor the survival of SCLC cells by preventing apoptosis in an unfavorable microenvironment (Masuda *et al*., 2001).

Tumor suppressor genes in the 3p region

Allelic loss involving the short arm of chromosome 3 is one of the most frequent genetic alterations in both SCLC (>90%) and NSCLC (~70%). Extensive study of the 3p alterations by many groups has led to the identification of at least four separate consensus regions of allelic loss; 3p25-p26, 3p21-p22, 3p14, and 3p12 (Hibi *et al*., 1992; Latif *et al*., 1992). In addition, recent high-resolution allelotyping indicated the presence of at least eight distinct sites with frequent LOH (Wistuba *et al*., 2000). It has been shown that allelic loss of 3p is detectable even in histologically normal or mildly abnormal epithelium in lung cancer patients and healthy (current and former) smokers (Mao *et al*., 1997; Wistuba *et al*., 1997). It is of note that the frequency and extent of 3p allelic loss increase progressively along with the increasing severity of histopathologic changes of preneoplastic/preinvasive lesions. These findings suggest that multiple tumor suppressor genes may reside in the 3p regions and that their inactivation may cumulatively occur due to insults imposed by cigarette smoking, leading to the development and progression of lung cancer.

Indeed, several putative tumor suppressor genes have been identified in homozygously deleted regions in lung cancer cell lines. The *FHIT* (*fragile histidine triad*) gene encoding a diadenosine triphosphate hydrolase was identified as a tumor-suppressor gene emcompassing the most frequent fragile site at 3p14.2 (Sozzi *et al*., 1996; Croce *et al*., 1999). FHIT inhibits tumor growth, when introduced into cancer cells with a *FHIT* gene alteration, through the induction of apoptosis and cell cycle arrest (Siprashvili *et al*., 1997; Sard *et al*., 1999). Mice with a targeted disruption of the *Fhit* gene have been shown to be highly susceptible to carcinogens (Zanesi *et al*., 2001). In addition to structural disruptions, as are expected from its location at the fragile site, an epigenetic mechanism leading to the loss of FHIT expression has also been suggested to play a role (Zochbauer-Muller *et al*., 2001). Loss of FHIT expression is detected in virtually all SCLC, while loss of FHIT expression is present more frequently in squamous cell carcinoma than in adenocarcinoma (Croce *et al*., 1999; Geradts *et al*., 2000; Wistuba *et al*., 2000). The association between loss of FHIT expression and smoking history has been reported in lung cancer and pre-neoplastic lesions (Sozzi *et al*., 1997; Tomizawa *et al*., 1998; Tseng *et al*., 1999). The *ROBO1/DUTT1* gene was isolated from a homozygously deleted region at 3p12-13 (Sundaresan *et al*., 1998), and shown to be a human homologue of the

Drosophila axon guidance receptor, *Roundabout* gene, which may function as a receptor of the Slit family. It is interesting that analysis of the *Robo1*/*Dutt1*-deficient mice revealed its involvement in bronchial development (Xian *et al*., 2001).

Allelic loss in the 3p21.3 region is among the earliest changes in lung cancer development, suggesting the presence of a tumor suppressor gene(s) important for the initiation of lung carcinogenesis (Wistuba *et al*., 2000). Two homozygously deleted regions at 3p21.3 have been identified in lung cancer cell lines. Genomic DNA contigs covering the homozygously deleted regions were cloned and sequenced, resulting in the identification of several candidate genes for the putative tumor suppressor gene (Ishikawa *et al*., 1997; Lerman and Minna, 2000). Among these candidate genes, *RASSF1* encoding a protein with a Ras-association domain as well as with homology to the Ras-effector Nore1 frequently shows loss of expression of the A isoform (*RASSF1A*) especially in SCLC due to the epigenetic mechanism of DNA hypermethylation of its promoter region. Introduction of exogenous RASSF1A inhibits cell growth and tumor formation of lung cancer cell lines (Dammann *et al*., 2000; Burbee *et al*., 2001; Agathanggelou *et al*., 2001). To date, rare genetic alterations have been identified in *RASSF1* as well as in other candidate tumor suppressor genes at 3p21.3 including *BLU*, *NPR2*/*Gene21*, *FUS1*, *HYAL1* (*hyaluronidase*), *FUS2*, and *SEMA3B* (*semaphorin 3B*) (Tomizawa *et al*., 2001). Notably, loss or a reduced level of expression has been clearly shown in *CACNA2D2* (*voltage-dependent calcium channel α2/δ subunit 2*), *SEMA3B*, *BLU*, and *HYAL1* at the 630 kb homozygously deleted region at 3p21.3 (Lerman and Minna, 2000). However, it remains to be elucidated which gene or genes are playing a decisive role in lung carcinogenesis, and why so many candidate tumor suppressor genes with rare genetic changes and frequent epigenetic inactivations exist in this particular chromosome region.

TGF-β signal pathway (TGF-β type II receptor (TGFBR2), 3p22; Smad2 (MADH2), 18q21.1; Smad4 (MADH4), 18q21.1)

TGF-β strongly inhibits the proliferation of normal epithelial cells including bronchial and peripheral lung epithelial cells through the induction of CDK inhibitors (p15INK4B, p21CIP1, p27KIP1) (Massague *et al*., 2000; Miyazono *et al*., 2000), as well as partly through down-regulation of MYC (Seoane *et al*., 2001). TGF-β signaling is known to be impaired by the activation of the MAPK cascade and NF-κB pathway, implying the presence of cross-talk between the signaling pathways. Lung cancer cell lines often show unresponsiveness to TGF-β signal, while expression profiling analysis has demonstrated that expression of the *TGFβRII* is a characteristic feature of normal lung tissues when compared with lung cancer specimens (Bhattacharjee *et al*., 2001). Although the intracellular mediators of TGF-β signaling, Smad4 and Smad2, are indeed mutated in lung cancer (Nagatake *et al*., 1996; Uchida *et al*., 1996), the low frequencies of these alterations indicated that *Smad4* and *Smad2* mutations do not account for the frequent TGF-β insensitivity, raising the possibility that additional molecules are involved. Indeed, it was recently reported that the expression of *TGFβRII* is frequently lost in lung cancers due to an epigenetic mechanism involving histone deacethylation and altered chromatin conformation (Osada *et al*., 2001). Genetic alterations of *TGFβRII* such as frame-shift mutations, as have been shown to be relatively frequent in colorectal cancers with the microsatellite instability phenotype, are rarely observed in lung cancers, perhaps being consistent with a lack of marked microsatellite instability in lung cancers (Tani *et al*., 1997; Takenoshita *et al*., 1997).

Cell cycle checkpoints and DNA repair mechanism

Cell cycle checkpoints induce arrests/delays of cell cycle progression and provide sufficient time for DNA repair, if possible, thereby protecting cells against carrying over damaged DNA. Although the mechanism to sense DNA damage is not yet fully understood, double-strand DNA breaks activate a series of signal transducing reactions, in which the ATM and ATR kinases play central roles by phosphorylating several key molecules including CHK1, CHK2, p53, BRCA1 and NBS1, leading to further downstream signaling and cell cycle arrest/delay at G1, S, and G2 (Figure 3). Frequent genetic alterations of the p53 gene impair the G1 checkpoint in the majority of lung cancers, while the G2 checkpoint impairment was recently shown to be frequently impaired specifically in SCLC (Konishi *et al*., 2002). In this connection, it is of note that *CHK2* is infrequently mutated in lung cancer (Haruki *et al*., 2000), while loss of *14-3-3σ* due to hypermethylation was recently shown to be frequent in SCLC (Osada *et al*., 2002).

Alterations in the number of chromosomes are very frequently observed in lung cancer, while chromosomal instability has been shown to be a common feature of lung cancer cells (Haruki *et al*., 2001). While the mitotic spindle checkpoint is involved in assuring a properly ordered chromosome segregation by preventing cells with an unattached kinetochore from entering into mitosis, lung cancer cell lines exhibit frequent impairment of this checkpoint (Takahashi *et al*., 1999). Mutations of the *BUB* and *MAD* genes, essential components of the mitotic-spindle checkpoint, have been detected in lung cancers (Nomoto *et al*., 1999; Yamaguchi *et al*., 1999; Sato *et al*., 2000), albeit at low frequencies. Cahill *et al*. (1998) reported that defects in the mitotic checkpoint are closely associated with the presence of chromosome instability in colorectal cancers, but there appear to be additional alterations in lung cancers, since there are examples of chromosome instability without mitotic checkpoint impairment (Haruki *et al*., 2001). In addition, p53 alterations may also be indirectly involved in the induction of

chromosomal numerical alterations, perhaps due to consequential defects in the post-mitotic checkpoint function (Haruki *et al*., 2001).

Other tumor suppressor genes

Many tyrosine and serine/threonine kinases are involved in growth-promoting pathways, while several phosphatases negatively regulate these pathways. PTEN/MMAC1 (phosphatase and tensin homolog/mutated in multiple advanced cancers 1) functions as a phosphoinositide 3-phosphatase that negatively regulates Phosphatidylinositol-3 kinase (PI3K)/AKT signaling, and has been suggested to be involved in the induction of anoikis and inhibition of cell migration (Yamada and Araki, 2001; Nadav and Katz, 2001). Although the chromosomal assignment of the *PTEN/MMAC1* gene (10q23.3) coincides with a frequently deleted region in many malignancies including lung cancer, somatic mutations or homozygous deletions of the *PTEN/MMAC1* gene are present in a relatively small subset of cell lines ($\sim$10%) and primary specimens (0$\sim$5%) of lung cancer (Forgacs *et al*., 1998; Yokomizo *et al*., 1998; Kohno *et al*., 1998; Cagle *et al*., 1997; Okami *et al*., 1998).

The 11q23 region is one of the most frequent targets for chromosomal deletions in a variety of cancers including lung cancer, and has been suggested to harbor at least two putative tumor suppressor genes (Wang *et al*., 1999). *TSLC1/IGSF4* encoding an immunoglobulin superfamily member was recently cloned from a genomic fragment corresponding to 11q23.2, based on its functional competency in the suppression of the tumorigenicity of human A549 and mouse Lewis lung cancer cell lines (Murakami *et al*., 1998). Although *TSLC1* appears to carry mutations rather infrequently, expression of TSLC1 has been shown to be frequently reduced by DNA hypermethylation of its promoter region (Kuramochi *et al*., 2001). Another putative tumor suppressor gene at 11q23, *PPP2R1B* encoding the β isoform of protein phosphatase 2 regulatory subunit A, has also been shown to be mutated in a fraction of cell lines (15%) and primary specimens (6%) of lung cancers (Wang *et al*., 1998b). The *PPP1R3* gene residing at 7q31, which encodes a protein phosphatase 1 regulatory (inhibitor) subunit 3A, has also been shown to exhibit infrequent mutations in NSCLC cell lines (15%) as well as in primary lung cancer tissues (5%) (Kohno *et al*., 1999).

Oncogenes and growth-promoting signals

Cell proliferation is positively regulated by several growth-promoting pathways including MAP kinase cascades as well as PI3K–AKT, Phospholipase C (PLC)–PKC, and NF-κB pathways (Figure 4). These pathways are functionally associated with each other. For example, a growth factor-mediated signal may diverge through a tyrosine kinase receptor into several pathways such as the RAS–RAF–MAPK, PI3K–AKT, and PLC–PKC pathways, whereas NF-κB is convergently activated by several distinct stimuli, including growth factors, cytokines, and extra-cellular matrix (ECM) attachment. Therefore, one alteration of a component of a growth-promoting pathway may aberrantly affect several signaling pathways, resulting in the broad range of regulatory changes of cell proliferation.

Anchorage-independent growth and resistance to anoikis is thought to be characteristic to cancer cells including lung cancer cells. The signaling of attachment to ECM is mediated by integrins, and activates several pathways including RAS–RAF1, JNK–AP-1, and PI3-K–AKT, and Rho family-PAK signaling pathways. The integrin signaling sustains ERK activation by growth factor stimuli (Roovers *et al*., 1999), suggesting extensive cross-talk between growth factor receptors and integrin signaling pathways (Figure 4) (Danen and Yamada, 2001; Frisch and Screaton, 2001). Anchorage-independent growth of lung cancer cell lines was shown to be inhibited by the induction of a dominant negative JNK or IκBβ (Xiao and Lang, 2000; Jiang *et al*., 2001), suggesting that the activation of JNK–AP-1 and AKT–NF-κB pathways is involved in the acquisition of anchorage-independent phenotype in lung cancer. Many alterations thus far identified in lung cancer may be involved in the activation of these JNK–AP-1 and AKT–NF-κB pathways. For example, activating mutation of the RAS genes, autocrine secretion of members of the EGF family, and overexpression of the EGF receptor are frequently observed in lung cancer, which conceivably activates these pathways. Activation of PI3-K signaling to AKT may also be facilitated by the presence of mutations in the PTEN or PP2A gene in lung cancer as discussed above, while constitutive activation of AKT is frequently detected in $>$80% of NSCLC cell lines, which may also play a role in the promotion of cell survival (Brognard *et al*., 2001).

The elucidation of complex divergent and convergent networks of these growth-promoting pathways should lead not only to a better understanding of the fundamental functional interaction of oncogenes, but also to the development of novel preventive and therapeutic approaches by manipulating functions of a key-molecule of the network.

The RAS gene family

The RAS family consists of three proto-oncogene members, KRAS (KRAS2), HRAS, and NRAS, which bind to GTP in the active state, and return to an inactive GDP-interacting state through their intrinsic GTPase activity (Moodie *et al*., 1993). A single amino acid substitution at codon 12, 13, or 61 affects the GTPase activity, resulting in the accumulation of an active GTP-bound form and the constitutive activation of downstream targets, the RAF1/MAPK pathway. The activation of RAF1 induces transcription of

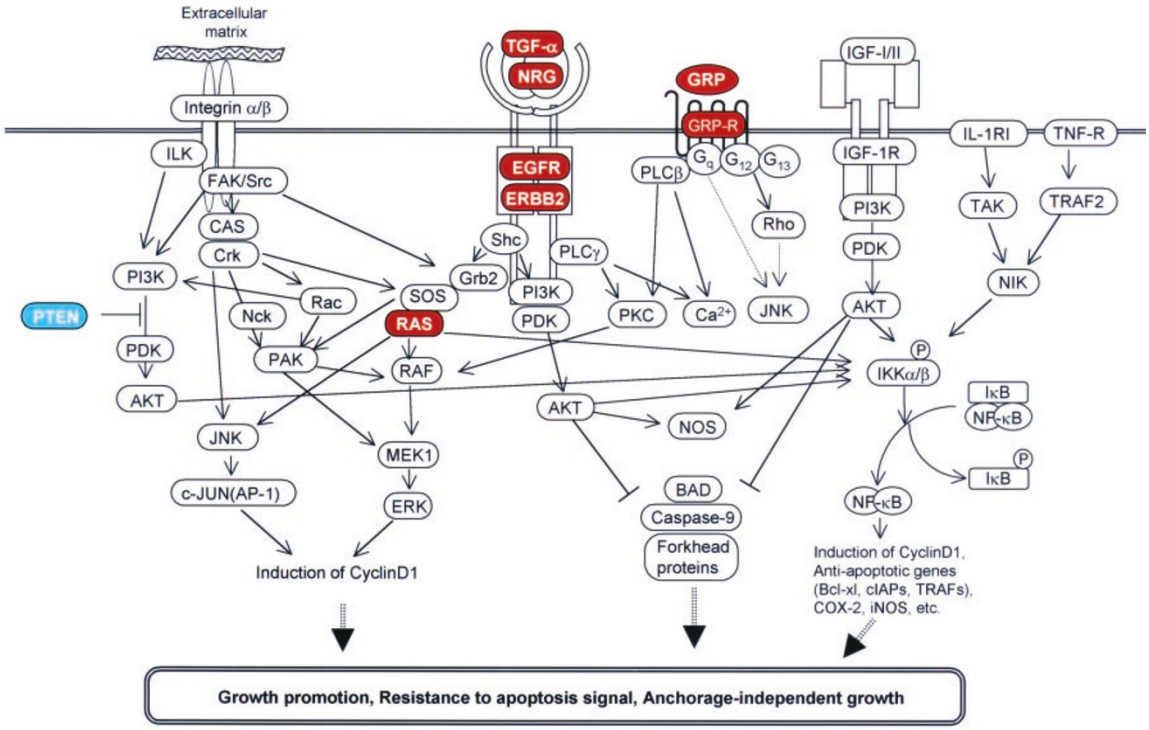

Figure 4 Activation of growth promoting pathways in lung cancer. The cell proliferation is positively regulated by several growth-promoting pathways including MAPK cascades as well as PI3K–AKT, PLC–PKC, and NF-κB pathways. Growth factor-mediated signals may diverge through a tyrosine kinase receptor into several pathways such as the RAS–RAF–MAPK, PI3K–AKT, and PLC–PKC pathways. NF-κB is convergently activated by several distinct stimuli including growth factors, cytokines, and extracellular matrix attachment. The PI3K–AKT signaling phosphorylates and inhibits several apoptosis-inducing genes. Autocrine secretion of the EGF family and/or overexpression of the EGF receptor family are frequently observed in lung cancer, which conceivably activates these pathways. In SCLC, autocrine activation of the GRP-mediated signaling is also frequently present, leading to activation of the ERK and JNK cascades. Stimulatory signalings via members of the EGF receptor family and various integrins coordinately promote cell proliferation (anchorage-dependent growth), while anchorage-independent growth may be associated with the activation of JNK–AP-1 and AKT–NF-κB pathways

several growth-promoting genes including those for *CyclinD1*, *MYC*, and growth factors mainly via the MAPK cascade (Schulze *et al*., 2001). Of particular note is that oncogenic *RAS* mutants also induce cell cycle arrest through induction of ARF, p16INK4A, and p53 expression in normal fibroblast cells (Palmero *et al*., 1998). This may in turn explain the frequent simultaneous occurrence of *KRAS* mutations and alterations of the p16INK4A/p14ARF and/or p53 pathway in cancer cells, and suggests the occurrence of *RAS* mutations in an ordered multi-step manner during the carcinogenic process. Although there has been considerable controversy over whether *KRAS* mutations are present in pre-malignant or normal-appearing lung tissues (Sugio *et al*., 1994; Urban *et al*., 2000), *KRAS* mutations are reportedly present in 25–40% of AAH lesions (Westra *et al*., 1996; Cooper *et al*., 1997). Among the *RAS* family members, the *KRAS* gene is most frequently affected in lung cancers, usually at codon 12 (Slebos *et al*., 1990; Mills *et al*., 1995), while mutations of *HRAS* or *NRAS* are very rare (~1%) in lung cancers. *KRAS* mutations are predominantly G to T transversions, implying the possible involvement of DNA adduct formation due to exposure to tobacco smoke in their occurrence (Rodenhuis and Slebos, 1990). *KRAS* mutations have been shown to be significantly associated with a shortened survival in surgically treated patients (Slebos *et al*., 1990). The *KRAS* mutations occur almost exclusively in adenocarcinoma, showing a strong association with a cytologic subtype of adenocarcnima, goblet cell type (Tsuchiya *et al*., 1995). The reason for the histologic type-specific involvement of *RAS* mutations in adenocarcinoma has not yet been elucidated.

MYC oncogenes

MYC protein forms a heterodimer with MAX and transactivates the cell cycle-regulating genes. MYC promotes cell cycle progression probably by several mechanisms. MYC represses p15INK4B through an inhibitory association with Miz-1, which induces p15INK4B expression (Staller *et al*., 2001; Seoane *et al*., 2001). The CDK inhibitor p27KIP1 is also down-regulated by MYC, because MYC induces the expression of Cyclin kinase sub-units (CKS) (Coller *et al*., 2000), which is required for ubiquitination of p27KIP1 by Skp2 (Spruck *et al*., 2001; Ganoth *et al*.,

2001). MYC induces CyclinD1/D2 expression and accumulation of CyclinD–CDK4 complex, which sequesters p21CIP1 and p27KIP1 and leads to the release of active CyclinE–CDK2 and cell cycle progression (Perez-Roger *et al*., 1999). Recent reports based on microarray analysis showed that MYC induced the expression of CyclinD2 and CDC2, and repressed the CDK inhibitor p21CIP1 (Coller *et al*., 2000; Guo *et al*., 2000).

Amplification of one of the members of the *MYC* gene family is detectable in 25–30% of SCLC cell lines as well as in 5–15% of primary tumor specimens, while a significantly higher level of expression of one of the members is detectable in virtually all SCLC cell lines (Little *et al*., 1983; Richardson and Johnson, 1993; Johnson, 1996). Thus, the vast majority of SCLC appear to have both p53 mutations and MYC overexpression, which may be consistent with the notion that inactivation of the ARF/MDM2/p53 pathway is required for tumorigenicity induced by MYC (Vonlanthen *et al*., 2001), which is known to activate the ARF/MDM2/p53 pathway (Zindy *et al*., 1998). In addition, cell lines and primary specimens of NSCLC exhibit *MYC* gene amplification and overexpression yet at lower frequencies (~30% and 8%, respectively), while overexpression of the *MYC* gene is observed frequently in cell lines (~70%) and primary specimens (~50%) of NSCLC (Little *et al*., 1983; Cline and Battifora, 1987; Gazzeri *et al*., 1990, 1994; Johnson, 1996). As for the relationship of *MYC* gene alterations to clinical status and course, a higher prevalence of *MYC* gene amplification has been reported in lung cancer cell lines established from treated than untreated patients' tumors, and *MYC* amplification in treated patients' tumor cell lines is associated with a shortened survival (Johnson *et al*., 1987). The DNA amplification of the *MYC* family is more frequently observed in cell lines (25–30%) than in primary tumors, suggesting a possible *in vitro* selective growth advantage of having an amplified *MYC* gene. In this connection, Barr *et al*. (2000) recently suggested that the amplification of *MYC* in lung cancer cell lines may be partly an artifact of selection for growth *in vitro*, based on the findings that overexpression of MYC experimentally enhanced the cell proliferation and soft agar cloning efficiency of SCLC cells, but decreased tumor formation in athymic mice through down-regulation of VEGF. The mechanism of MYC overexpression without DNA amplification is yet to be elucidated, but loss of transcriptional attenuation of the *MYC* and *MYCL* genes (Krystal *et al*., 1988) and antisense mRNA expression of the *MYCN* gene have been suggested to be involved (Krystal *et al*., 1990).

Growth factors and their receptors

The ERBB family consists of four receptor-type tyrosine kinases, EGFR (ERBB), ERBB2 (HER-2/neu), ERBB3, and ERBB4, for which several specific ligands have been identified. EGF, TGF-α, and amphiregulin (AR) are specific for EGFR, while neuregulins (NRG)/heregulins bind to ERBB3 or ERBB4. ERBB2 forms a heterodimer with other members of the ERBB family and enhances their signaling (Slichenmyer and Fry, 2001). The specific interactions of these ligands and receptors stimulate several signaling pathways such as RAS–RAF–MAPK, PI3K–AKT, and PLC–PKC, mediating growth-stimulating signals (Schlessinger, 2000). In contrast to its frequent gene amplification in breast cancers, EGFR is overexpressed without gene amplification in a large proportion of lung cancers (70% of squamous cancers and 40% of adenocarcinomas) (Rusch *et al*., 1993; Rachwal *et al*., 1995). The EGFR type III mutation (inframe deletion within the extracellular domain), which is reportedly present in 16% of NSCLC, has been suggested to be a tumor-specific antigen potentially useful for targeting cancer cells (Garcia de Palazzo *et al*., 1993). Overexpression of ERBB2 is also present in 30% of lung cancers (Kern *et al*., 1990; Weiner *et al*., 1990), although gene amplification is very rare (~3%) (Schneider *et al*., 1989; Shiraishi *et al*., 1989). It has been shown that ERBB3 overexpression is detectable in 20% of NSCLC, showing an association with a poor prognosis (Yi *et al*., 1997). Overexpression of TGF-α is present in 60% of NSCLC, suggesting the existence of a possible autocrine/paracrine stimulatory loop in lung cancer (Yoneda and Boucher, 1993; Rusch *et al*., 1993). High-level expression of AR was reported to occur in over 40% of lung cancers and has been suggested to be associated with a poor prognosis (Fontanini *et al*., 1998), while expression of NRG has also been reported in lung cancer cell lines (al Moustafa *et al*., 1999).

Angiogenic factors

Angiogenesis is thought to play an important role in tumor progression and metastasis, and is positively and negatively regulated by many cytokines and growth factors, including VEGFs, angiopoietins (Angs), PDGF, bFGF, IL-8, TGF-β, and TGF-α. Two distinct families of endothelial cell-specific receptor tyrosine kinases, the VEGFR and TIE families, have been recognized and thought to promote angiogenesis by mediating signals elicited by binding of the VEGFs and Angs, respectively (Karkkainen and Petrova, 2000; Jones *et al*., 2001). VEGFR2 (KDR/Flk-1) acts as a major receptor transducing VEGF's effects in endothelial cells, whereas VEGFR1 (Flt-1) plays a negative regulatory role by sequestering VEGF (Karkkainen and Petrova, 2000). VEGFR-3 (Flt4) mediates VEGF-C/D signals and promotes lymphangiogenesis (Karkkainen and Petrova, 2000). Among the TIE family, TIE2 (TEK) interacts with Angs, while the ligand of TIE1 remains to be identified. Genetic lesions such as gene amplification involving VEGFs, Angs and their respective receptors have not been reported in lung cancer, but high-level expression of VEGF is detectable in about 50% of lung cancers, showing an association with a higher microvessel density (O'Byrne *et al*., 2000; Yuan *et al*., 2000). It has been suggested that VEGF

expression is induced by HIF-1α responding to hypoxia in lung cancer tissues (Giatromanolaki *et al*., 2001), while COX-2 and NOS2-mediated stimuli have also been postulated as leading to marked VEGF expression in NSCLC (Marrogi *et al*., 2000). It remains controversial whether high level of VEGF expression and/or a high microvessel density correlate with a poor prognosis (Fontanini *et al*., 1999; Yano *et al*., 2000; Decaussin *et al*., 1999; Marrogi *et al*., 2000). VEGF-C promotes lymphangeogenesis through the activation of VEGFR-3-mediated signals, and is expressed in about 40% of NSCLC, raising the possibility of its involvement in lymphatic metastasis (Kajita *et al*., 2001). Recently, frequent expression of Ang-1 and TIE2 in NSCLC was reported, suggesting their possible involvement in angiogenesis in lung cancers (Takahama *et al*., 1999; Hatanaka *et al*., 2001).

Telomerase

Telomere maintenance is a critical component of immortality due to the inability of DNA polymerases to completely replicate the end of the chromosomal DNA. It has been suggested that inactivation of p16INK4A/RB pathways may be involved in the senescence-overriding process, causing cells to continue to lose telomeric DNA, which finally triggers removal by p53-mediated apoptosis (Kiyono *et al*., 1998). Loss of p53 function allows pre-malignant cells to continue proliferating and subsequently reach a second barrier, crisis, which is characterized by chromosomal instability, end-to-end fusions, and catastrophic cell death. Telomerase activation and subsequent stabilization of the telomeric DNA is thought to be essential for cells to escape from this crisis in order to proliferate immortally (Artandi and DePinho, 2000).

Indeed, the activation of telomerase is frequently observed in lung cancers (100% of SCLC and 80% of NSCLC) (Hiyama *et al*., 1995), while telomerase activity was shown to be correlated with cell proliferation and pathological progression in NSCLC (Albanell *et al*., 1997). In addition, telomerase activity is also detectable in pre-cancerous tissues, suggesting the early involvement of up-regulation of this enzyme. Although the precise molecular mechanism involved in the activation of telomerase remains rather obscure, Myc has been suggested to play a role by directly transactivating *hTERT* (Wang *et al*., 1998a; Wu *et al*., 1999). Interestingly, microcell transfer experiments have revealed that a gene or genes that represses expression of *hTERT* may be present at 3p, which is among the most frequently altered regions in lung cancer (Ohmura *et al*., 1995; Cuthbert *et al*., 1999).

Conclusion

Accumulating evidence clearly indicates that perturbation of the integrity of integrated signaling networks, which positively or negatively regulate various cellular processes to maintain homeostasis of the lung, leads to the carcinogenesis and progression of lung cancer. In the end, the accumulated genetic and epigenetic alterations are thought to similarly confer various capabilities on lung cancer cells, including an escape from growth inhibitory signals as well as from excess shortening of telomeres, resistance to apoptosis, sustained stimuli for proliferation and angiogenesis, and invasive and metastatic characteristics. The chronological order and catalogue of genes required to fully transform normal epithelial cells may, however, vary to a certain extent among various histological types of lung cancers or even within a given histological subtype. Recent expression profiling analyses of lung cancers demonstrated histological type-specific clustering of SCLC and squamous carcinoma and indicated heterogeneity of adenocarcinoma, showing a relationship with clinical outcome (Garber *et al*., 2001; Bhattacharjee *et al*., 2001). In the coming decade, ample information on the human genome sequence as well as emerging new technologies including sophisticated informatics will provide for a complete picture of lung cancer biology and lead to a revolution in the prevention, diagnosis, and treatment of this fatal disease.

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