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Pharmacogenetics of Anticancer Drug Sensitivity in Non-Small Cell Lung Cancer

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***Abstract*——In mammalian cells, the process of malignant transformation is characterized by the loss or down-regulation of tumor-suppressor genes and/or the mutation or overexpression of proto-oncogenes, whose products promote dysregulated proliferation of cells and extend their life span. Deregulation in intracellular transduction pathways generates mitogenic signals that promote abnormal cell growth and the acquisition of an undifferentiated phenotype. Genetic abnormalities in cancer have been widely studied to identify those factors predictive of tumor progression, survival, and response to chemotherapeutic agents. Pharmacogenetics has been founded as a science to examine the genetic basis of interindividual variation in drug metabolism, drug targets, and transporters, which result in differences in the efficacy and safety of many therapeutic agents. The traditional pharmacogenetic approach relies on studying sequence variations in candidate genes suspected of affecting drug response. However, these studies have yielded contradictory results because of the small number of molecular determinants of drug response examined, and in several cases this approach was revealed to be reductionistic. This limitation is now being overcome by the use of novel techniques, i.e., high-density DNA and protein arrays, which allow genome- and proteome-wide tumor profiling. Pharmacogenomics represents the natural evolution of pharmacogenetics since it addresses, on a genome-wide basis, the effect of the sum of genetic variants on drug responses of individuals. Development of pharmacogenomics as a new field has accelerated the progress in drug discovery by the identification of novel therapeutic targets by expression profiling at the genomic or proteomic levels. In addition to this, pharmacogenetics and pharmacogenomics provide an important opportunity to select patients who may benefit from the administration of specific agents that best match the genetic profile of the disease, thus allowing maximum activity.**

I. Introduction

The aim of this review is to examine the current understanding of the influence that the genetic profile of non-small cell lung cancer, the most frequent cause of cancer death in humans in the Western world, may have on the effect of chemotherapeutic agents. The application of the principles of pharmacogenetics by the use of novel techniques may lead to increasing predictability of drug response of the disease, with the aim of targeted therapeutic intervention.

II. Clinical Relevance and Management of Non-Small Cell Lung Cancer

Lung cancer is a leading cause of mortality among men and women in the Western world, with 170,000 deaths per year. This exceeds the sum of the next three leading causes of death due to breast, colon, and prostate cancer. There are over one million deaths worldwide due to malignant tumors of the lung, making it an epidemic disease (Jemal et al., 2002). Lung cancer is a deadly illness because of the low proportion of subjects (~15%) that are still alive 5 years after the initial diagnosis. Patients with stage I disease (T1–2, N0, M0) may be cured by optimal treatment, and 70% of them may achieve a 5-year survival; unfortunately, most subjects present with advanced disease, and this condition adversely affects survival (Cortes-Funes, 2002; Ferreira et al., 2002).

From a histological point of view, lung cancer is classified into non-small cell (NSCLC[1]) and small cell lung cancer (SCLC); 80% are NSCLCs, including adenocarcinomas, squamous cell (epidermoid), and large cell carcinomas, and 20% are SCLCs (Rom et al., 2000).

[1] NSCLC, non-small cell lung cancer; ABC, ATP-binding cassette; Akt, AKR mouse T-cell lymphoma; ANA, abundant in neuroepithelium area; APC/MCC, adenomatous polyposis coli/mutated in colon cancer; BAT-26, mononucleotide microsatellite containing a 26-repeat adenine tract; CAAX, cysteine, aliphatic amino acid and any amino acid; cdc2, cell division control kinase 2; cdc25B, cell division control kinase 25B; CDK, cyclin-dependent kinase; CDKN2A, cyclin-dependent kinase inhibitor 2A; CGH, comparative genomic hybridization; cM, centimorgan; Cox-2, type-2 inducible isoform of cyclooxygenase; CpG, cytidine phosphate guanosine; DAPK, death-associated protein kinase; DMs, double minute chromosomes; DNA-MTase, DNA-methyltransferase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EMAST, elevated microsatellite alterations at selected tetranucleotide repeats; ERCC1, excision repair cross-complementing 1 gene, ERK, extracellular signal-regulated kinase; FHIT, fragile histidine triad; FISH, fluorescence in situ hybridization; GML, glycosyl-phosphatidyl-inositol-anchored molecule-like protein; GSTP1, glutathione *S*-transferase P1 isoform; GDP/GTP, guanosine diphosphate/triphosphate; HDAC, histone deacetylase; hMLH1, human MutL homolog-1; hMSH3, human MutS homolog-3; HSRs, homogeneously staining regions; hTERT, human telomerase reverse transcriptase; IC_{50}, 50% inhibitory concentration; IC_{80}, 80% inhibitory concentration; ICE, interleukin-1β-converting enzyme; LOH, loss of heterozygosity; LRP, vault-transporter lung resistance protein; LRP-DIT, lipoprotein receptor-related protein-deleted in tumors; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MAPK, mitogen-activated protein kinase; MDM2, mouse double minute 2; MDR1, multidrug resistance 1 gene; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MGMT, O^6-methylguanine-DNA methyltransferase; MMP, matrix metalloproteinase; MRP, multidrug resistance-associated protein; NER, nucleotide excision repair; PCNA, proliferating cell nuclear antigen; P-gp, P-glycoprotein; PI3K; phosphatidylinositol 3-kinase; PKB, protein kinase B; PTEN/MMAC1, phosphatase and tensin homolog deleted on chromosome 10/mutated in multiple advanced cancers 1; RAR, retinoic acid receptor; RASSF1, RAS effector homolog 1; RB, retinoblastoma tumor suppressor gene; RER+, replication-error-type instability; RPL14, ribosomal protein L14 gene; RT-PCR, reverse-transcriptase polymerase chain reaction; RXR, retinoid X receptor; SCLC, small cell lung cancer; SELDI-TOF, surface-enhanced laser desorption/ionization time-of-flight; SNP, single nucleotide polymorphism; SROs, short regions of the overlap of the deletions; TGF-α, transforming growth factor-α; TGF-β, transforming growth factor-β; TGFβ-RII, transforming growth factor-β type II receptor; TNM, tumor, lymph node, metastasis staging system; TRAIL-R2, tumor necrosis factor-related apoptosis-inducing ligand receptor 2; TSLC1, tumor suppressor lung cancer 1;VEGF, vascular endothelial growth factor; VNTR, variable number of tandem repeats.

Surgery is the recommended treatment; chemotherapy and radiotherapy have a role either as a part of a treatment strategy to cure locally advanced disease or as a palliative therapy for metastatic tumors. In patients with stages I and II NSCLC (T1–2, N1, M0) who cannot be treated by surgery because of nontumor-related comorbidity, radiotherapy is the therapeutic approach of choice. With standard radiotherapy, the survival at 3 and 5 years of patients with stage I disease is about 30 to 40% and 10 to 30%, respectively (Bonnet et al., 2001; Jeremic et al., 2002), while the 2-year survival is 20% for stage II disease. The prognosis of stage IIIA (T3, N0–1, M0 or T1–3, N2, M0) and IIIB (any T4 or any N3, M0) NSCLC is dismal, although still curable in some cases. Survival at 5 years is about 5% in patients with N2 disease (involvement of ipsilateral or subcarinal mediastinal lymph nodes or ipsilateral supraclavicular lymph nodes), and 50% in patients with T3 disease (tumor involving the pleura, chest wall, diaphragm, or pericardium) without lymph node involvement. Patients with N3 (contralateral mediastinal hilar or supraclavicular lymph node involvement) or T4 tumors (invasion of mediastinal organs, malignant pleural effusion) are treated with palliative intent (Malayeri et al., 2001). Postoperative mediastinal radiation therapy has been shown to significantly reduce the risk of local relapse with no or little impact on survival (PORT Meta-analysis Trialists Group, 1998), while adjuvant chemotherapy is ineffective (Souquet and Geriniere, 2001). Preoperative chemotherapy followed by surgery and postoperative radiation therapy, in the case of incomplete resection, seems to be able to prolong disease-free and overall survivals in comparison with surgery alone (Rosell et al., 1994; Roth et al., 1994; Gandara et al., 2001; Rinaldi and Crinò, 2001). Combined chemo-radiotherapy has a strong rationale due to its potential synergistic effects, although the results for locally advanced inoperable disease are still controversial and its feasibility before surgery has been proven only in phase II trials, many of them including stage IIIA-B patients (Lau et al., 2001).

The median survival of patients with metastatic NSCLC treated with chemotherapy is in the range of 8 to 10 months. Standard chemotherapy consists of combination regimens containing cisplatin, carboplatin, paclitaxel, docetaxel, gemcitabine, vinorelbine, ifosfamide, and etoposide. However, recent randomized studies on more than 2200 patients failed to show major differences in response rates and survival among the combination of cisplatin + gemcitabine, cisplatin + vinorelbine, and cisplatin or carboplatin + paclitaxel or docetaxel (Table 1). According to the results reported, a response rate exceeding 40% cannot be expected, irrespective of what drug combination is administered; in addition to this, the survival rate of patients older than 70 years of age who are treated with chemotherapy not containing cisplatin is similar to that of younger patients (Alberola et al., 2001; Gridelli et al., 2001, 2002; Rodriguez et al., 2001; Scagliotti et al., 2001; Van Meerbeeck et al., 2001) (Table 1).

Chemotherapy has a role in the treatment of locally advanced and metastatic NSCLC, either as a part of a curative strategy or with palliative intent, although clinical response to chemotherapy is still unsatisfactory, particularly with respect to the complete response rate, which is still low. Therefore, a better selection of patients and the identification of predictive factors of sensitivity to chemotherapeutic agents are warranted.

Concerning locally advanced disease (stage IIIA-B), resectability criteria are not uniformly accepted; thus, the distinction between patients with resectable and unresectable tumor may be difficult. Preoperative induction chemotherapy provides a response rate of approximately 60% and a downstaging to resectable disease in 44 to 65% of subjects (Felip and Rosell, 2002). Surgically treated patients achieve a median survival of approximately 20 months and a long-term survival of 20 to 25%.

TABLE 1
Summary of selected randomized trials in advanced NSCLC

Authors	Patients	Chemotherapy	Response Rate	Median Survival	1-Year Survival	2-Year Survival
			%	*months*	%	%
Rodriguez et al., 2001	408	Cisplatin + docetaxel		10.9	47	21
	406	Carboplatin + docetaxel		9.1	38	16
	404	Cisplatin + vinorelbine		10	42	14
Scagliotti et al., 2001	201	Cisplatin + vinorelbine	30	9.5	37	
	201	Carboplatin + paclitaxel	32	9.9	43	
	205	Cisplatin + gemcitabine	30	9.8	37	
Van Meerbeeck et al., 2001	161	Paclitaxel + gemcitabine	27	6.9	26.5	
	160	Cisplatin + gemcitabine	26	8.8	33	
	159	Cisplatin + paclitaxel	31	8.1	36	
Alberola et al., 2001	166	Cisplatin + gemcitabine	43	8.7	35	
	176	Cisplatin + gemcitabine + vinorelbine	39	7.9	31	
	175	Gemcitabine + vinorelbine → ifosfamide + vinorelbine[a]	26	8.1	35	
Gridelli et al., 2001[b]	233	Vinorelbine	18.5	8.8	41	
	233	Gemcitabine	17.3	6.6	26	
	232	Gemcitabine + vinorelbine	20	7.6	31	

[a] Sequential treatment with two regimens.
[b] Patients older than 70 years.

Pathologically complete remissions are low and about 10% of patients will progress under induction chemotherapy (Rosell et al., 1994; Roth et al., 1994; Kumar et al., 1996; Rinaldi and Crinò, 2001). Induction chemotherapy followed by full-dose radiotherapy is suitable for patients with good performance status (Pottgen et al., 2002), and preoperative radio-chemotherapy is suitable for selected patients because of up to 10% morbidity and mortality (Thomas et al., 1999). In patients with incomplete resection, postoperative radiotherapy may be administered (Grossi et al., 2001; Pitz et al., 2002).

Radiotherapy is considered the standard treatment for unresectable tumors; a total dose of at least 60 Gy results in a survival of about 30% and 7% at 1 and 3 years, respectively (Cox et al., 1991; Baumann et al., 2001). A *meta*-analysis study showed that the addition of chemotherapy to radiotherapy has a marginal impact on survival, with a hazard ratio of 0.94 and a 2% absolute benefit at 2 and 5 years (Non-Small Cell Lung Cancer Collaborative Group, 1995). An RTOG trial provided evidence in support of simultaneous chemotherapy and radiotherapy compared to sequential treatment (median survival 17 versus 14.6 months), although the overall results cannot be considered satisfactory (Werner-Wasik et al., 2000). Finally, patient with stage IIIB disease may be administered chemotherapy for palliative intent.

The vast majority of patients with metastatic NSCLC (stage IV) die from disseminated cancer within two years of follow-up. Since the survival benefit is small and the prognosis is poor, the role of chemotherapy is doubtful and it is not recommended as a standard treatment in subjects in poor general condition (Non-Small Cell Lung Cancer Collaborative Group, 1995; Socinski et al., 2002). Standard chemotherapy mainly consists of combination regimens containing cisplatin; a 27% reduction in the risk of death has been reported, equivalent to an absolute improvement in survival of 10% or an increased median survival of 1.5 months, and a lower incidence of disease-related complications has been observed (Non-Small Cell Lung Cancer Collaborative Group, 1995; Manegold, 2001). Isolated symptomatic lesions, including bone metastases and spinal cord compression, are treated with radiotherapy. Second-line chemotherapy may be effective in some patients; docetaxel produces good results in cisplatin-treated patients (Fossella et al., 1995; Kim et al., 2002), while cisplatin-based chemotherapy after paclitaxel and gemcitabine has been reported to be effective only in 20% of those patients responsive to the first-line treatment and in none with refractory disease (De Pas et al., 2001).

The assessment of the prognosis of patients with lung cancer is essential for the choice of the best therapeutic option. The major clinical prognostic determinant in NSCLC is tumor extension; patients with advanced, unresectable NSCLC have a poor prognosis, with very few 5-year survivors and a median survival of less than 1 year. However, a large variability of clinical outcome characterizes this subset of patients, some of them surviving only a few weeks and some others several years. Many prognostic factors have been recognized and are currently being evaluated to support therapeutic decisions. In patients with surgically resected stage I NSCLC, the prognostic significance of a panel of tumor markers, including ErbB-1/epidermal growth factor receptor (EGFR), *HER-2/neu* (ErbB-2), Bcl-2, p53, and angiogenesis was evaluated. Statistical analysis demonstrated that tumor extension represented the most powerful prognostic factor for survival and time to recurrence, while increased EGFR expression was significantly associated with a poorer survival ($P = 0.02$); none of the other immunocytochemical markers was an independent predictive factor for survival (Pastorino et al., 1997). Furthermore, the immunohistochemical analysis of protein expression profiles of 216 patients with NSCLC demonstrated that the expression of nuclear oncoproteins fos and jun and of cyclin A were decreased in carcinomas of patients with long-term survival (Volm et al., 2002).

The subgroup of patients with metastatic NSCLC is heterogeneous, and the differentiation between patients with single or multiple metastases has prognostic relevance. Patients with a single metastasis, particularly in the absence of mediastinal lymph node involvement, have a better prognosis than patients with multiple distant sites of disease. When a single brain metastasis is the only site of first recurrence in patients free of extracranial disease, a surgical approach with brain tumor resection or stereotactic radiosurgery improves the quality of life and offers a chance of long-term survival, with a median survival of up to 27 months (Arbit et al., 1995; Granone et al., 2001). Together with pretreatment stage, performance status and weight loss are important prognostic factors in advanced NSCLC (Paesmans et al., 1995; Buccheri and Ferrigno, 2001). This reflects the tumor biological profile, which in turn translates into the aggressiveness of the disease. Moreover, patients with poor performance status and severe weight loss are unsuitable candidates for antitumor treatment and are more susceptible to severe medical complications. Other factors have a prognostic role in patients with NSCLC, although not always confirmed in retrospective analyses: among the others, it seems to be relevant to the male gender, the presence of clinical symptoms (i.e., cough and hemoptysis), and elevated neutrophil count (Paesmans et al., 1995; Martins and Pereira, 1999). Stages I–IIIA NSCLC are potentially resectable; however, patients belonging to these groups are highly heterogeneous with respect to their prognosis, since the 5-year survival rate is about 80% for stage I and 20 to 30% for stage IIIA. For stage I, important independent prognostic factors are the volume of primary tumor and pretreatment serum lactate dehydrogenase (Feld et al., 1997). Mediastinal lymph node involvement is an important adverse prognostic factor, and the strongest predic-

tor of long-term survival after surgery is the absence of mediastinal neoplastic spread. Patients with metastatic ipsilateral or subcarinal mediastinal lymph nodes or ipsilateral supraclavicular lymph nodes (N2) are nonetheless a heterogeneous subgroup. Moreover, N2 lymph node involvement has a different prognostic value if clinically or pathologically detected. Patients with preoperative evidence of N2 disease have a worse prognosis than patients with clinically undetectable involvement (Andre et al., 2000). Moreover, the number of metastatic mediastinal lymph nodes proved to be an independent prognostic factor and was related to a significant difference in overall survival of surgically treated stage IIIA NSCLC (Andre et al., 2000). Patients with single lymph node metastasis showed a longer median survival than patients with multiple lymph node involvement.

The spreading of tumor cells in the bone marrow of patients with clinically localized NSCLC may be detected by immunohistochemical analysis, and it is associated with a poor prognosis (Pantel et al., 1996; Osaki et al., 2002). Finally, histopathology has no prognostic value and the importance of tumor cell differentiation is controversial, while a poor prognosis is correlated with lymphatic and vascular invasion and the expression of mucin and a high mitotic index of cancer cells (Komaki et al., 1998).

III. Genetic Instability and Gene Dysfunction in Non-Small Cell Lung Cancer

Tumor evolution is a multistep process characterized by the loss of function of cellular mechanisms that control normal proliferation and differentiation. It is estimated that 10 to 20 genetic events, including the alteration of oncogenes and tumor-suppressor genes, will have occurred by the time a lung tumor becomes clinically evident (Tran et al., 1998). Genetic instability is the hallmark of cancer as a disease. It may be indicated by a variety of cellular features at the chromosomal and DNA levels. Evidence of DNA instability is represented by the incidence of point mutations, deletions/insertions, recombination, gene amplification, and microsatellite instability, while at the chromosomal level it consists of aneuploidy, translocations, deletions, sister chromatid recombinations, fragile sites, homogeneously stained regions, and double minute chromosomes (Sherbet and Lakshmi, 1997).

A. Gene Amplification

Oncogene amplification is frequently detected in human cancer and it is characteristic of solid tumors, including NSCLC. DNA amplification does not occur in normal cells and it is maintained in cancer cells as a result of selection. DNA amplification is observed with cytogenetic methods as double minute chromosomes (DMs) or homogeneously staining regions (HSRs), but more recent technologies, including fluorescence in situ hybridization (FISH) and comparative genomic hybridization (CGH), have substantially increased the ability to detect such alterations (Imreh et al., 1997; Grompe et al., 1998). DMs are episomal forms of amplified DNA that generally lack centromeres and are unequally distributed between daughter cells at mitosis. In contrast, HSRs are chromosomally integrated forms of amplified DNA. They represent either the replacement of the normal chromosome banding pattern with an extended region of homogeneous staining or the insertion of such a region into an otherwise normally banded chromosome (Grompe et al., 1998). DMs and HSRs tend to be mutually exclusive and are potentially interchangeable manifestations of amplified DNA; therefore, DMs can potentially integrate into distant chromosomal sites to generate heritable HSRs. DMs, and less frequently HSRs or a combination of both, are found in approximately 17% of NSCLC (Imreh et al., 1997).

The vast majority of oncogene amplifications found in human cancers affect the *myc* family; among them, *c-myc* is amplified in >50% of NSCLC and it correlates with the extent of lymph node metastasis (Kubokura et al., 2001; Salgia and Skarin, 1998). Less frequent gene amplification involves the *cdc25B* gene (40%) (Wu et al., 1998), cyclin D1 (5%), and the *EGFR* gene (5.9%) (Reissmann et al., 1999), and *HER-2/neu* (<2%) (Cox et al., 2001).

B. Gene Mutation

Mutations are DNA sequence alterations that may result in the disruption or abnormal activity of a gene or the encoded protein. These include gene rearrangements, deletions, insertions, and single-base changes. Nonsense mutations result in the appearance of a stop codon and premature termination during protein synthesis, missense mutations are single-base substitutions causing incorporation of an inappropriate amino acid into a protein, and frameshift mutations shift the reading frame of triplet codons in a gene during mRNA translation (Grompe et al., 1998). Mutations that cause the synthesis of structurally aberrant proteins usually occur within the coding region of the gene, while those that result in the production of abnormal amounts of the protein may affect the 1) transcriptional machinery, 2) regulatory regions (i.e., gene promoter), 3) RNA processing (i.e., splicing alterations in the 5′ untranslated region or 3′ adenylation signals), or 4) translational machinery that controls initiation, elongation, and termination of polypeptide chains (Grompe et al., 1998). At variance with a mutation, a DNA polymorphism is a sequence alteration stably expressed and found at a frequency of >1% in a given population. The simplest type is the single nucleotide polymorphism (SNP), a single base difference between genome sequences that occurs approximately every 1 kb in the human genome (Grompe et al., 1998; Danesi et al., 2001). Additional types of polymorphism are represented by the variable

number of tandem repeats (VNTR, minisatellites), multiple copies of short repeats of DNA sequences (0.1–10 kb) distributed along the human genome, and the microsatellite repeats, a simpler but more common variant of minisatellites, in which up to tetranucleotide repeats are reiterated in multiple copies (Grompe et al., 1998).

Although point mutations are more commonly associated with loss of function (i.e., the *TP53* gene), there are notable examples of activating point mutations in a cellular proto-oncogene; indeed, in approximately 30% of human NSCLCs, the *K-ras* oncogene is mutated (Noda et al., 2001). Mutations affecting the tumor-suppressor gene *TP53* may be associated with deregulation in telomerase activity, which in turn may be important in the process of lung tumorigenesis and low-grade differentiation in NSCLC (Maniwa et al., 2001). The simultaneous occurrence of *TP53* gene mutation and high telomerase activity may be relevant to the grade of malignancy in lung tumors (Maniwa et al., 2001). In NSCLC, inactivation by point mutation of the *CDKN2A* (cyclin-dependent kinase inhibitor 2A) gene, which encodes $p16^{INK4a}$ (p16 inhibitor of kinase 4a), is observed in smokers, whereas *CDKN2A* is inactivated in nonsmokers through promoter hypermethylation (Sanchez-Cespedes et al., 2001). Additional mutations observed in NSCLC involve the tumor necrosis factor-related apoptosis-inducing ligand-receptor 2 (*TRAIL-R2*) gene (10.6% mutations), mapped to chromosome 8p21–22 and encoding a cell-surface receptor involved in cell death signaling (Lee et al., 1999a), and the lipoprotein receptor-related protein-deleted in tumors (LRP-DIT), a tumor-suppressor gene that is inactivated by homozygous deletion or mutation in at least 40% of NSCLC cell lines and thus may play an important role in lung tumorigenesis (Liu et al., 2000).

C. Promoter Hypermethylation

Neoplastic cells simultaneously harbor diffuse genomic hypomethylation, more regional areas of hypermethylation, and increased DNA-methyltransferase (DNA-MTase) activity. Each component of this *methylation imbalance* may contribute to tumor progression. Main targets of the regional hypermethylation are the normally unmethylated CpG (cytidine phosphate guanosine) islands located in gene promoter regions. In particular, methylation of normally unmethylated sites in the promoter regions of tumor-suppressor and DNA-repair genes is correlated with loss of expression of these genes in cancer cell lines and primary tumors (Baylin and Herman, 2000). Methylation of the CpG islands in the O^6-methylguanine-DNA methyltransferase (MGMT) prevents gene transcription, and cells cannot repair the alkylation of O^6-methylguanine (Qian and Brent, 1997; Watts et al., 1997; Esteller et al., 1999a; Danam et al., 1999). Furthermore, in vitro treatment with demethylating drugs restores the expression of MGMT (Qian and Brent, 1997; Esteller et al., 2000).

DNA hypermethylation is associated with transcriptional repression and represents an alternative to coding region mutations for inactivation of tumor-suppressor genes, including *CDKN2A* ($p16^{INK4a}$). The hypermethylation of a promoter is an epigenetic phenomenon that leads to its inactivation or down-regulation of gene transcription (Baylin et al., 1998). CpG sequences are located in the promoter regions of about 50% of all human genes; in normal cells, unmethylated CpG islands are protected from methylation on flanking regions, while in neoplastic cells this protection is lost (Baylin et al., 1998; Wistuba et al., 2001). Promoter hypermethylation has relevance in the development of cancer, as it occurs at the level of tumor-suppressor genes (Costello et al., 2000; Wistuba et al., 2001). Promoter hypermethylation is frequently detected in NSCLC, and the number of CpG sequences that are methylated may be very large (up to 4500) (Costello et al., 2000). The hypermethylation of promoter regions of *CDKN2A* ($p16^{INK4a}$), death-associated protein kinase (DAPK), GSTP1 (glutathione *S*-transferase P1 isoform), and MGMT has been detected in 68% of NSCLC, but not in surrounding normal tissues. Moreover, 73% of patients with abnormal methylation patterns at the level of promoters also showed circulating DNA with abnormal patterns of methylation, while patients without methylation abnormalities did not show the same alterations in circulating DNA, thus suggesting that this finding is specific and may allow genetic testing that may be useful for treatment selection and diagnosis of disease recurrence (Esteller et al., 1999b). DAPK promoter hypermethylation is observed in 44% of patients with stage I NSCLC; this genetic abnormality predicts an adverse prognosis, as the 5-year survival after surgical resection is significantly poorer with respect to those patients without DAPK promoter hypermethylation (Tang et al., 2000). Aberrant methylation of the *CDKN2A* ($p16^{INK4a}$) tumor-suppressor gene has been detected in the early stage of NSCLC. *CDKN2A* ($p16^{INK4a}$) has a relevant role in NSCLC carcinogenesis, and its silencing has been detected at high frequency in invasive and in situ tumors. In particular, *CDKN2A* ($p16^{INK4a}$) methylation is detected in 17% of basal cell hyperplasia, in 24% of squamous metaplasia, and in 50% of in situ carcinomas; this proportion further increases (75%) in in situ carcinoma adjacent to invasive squamous cell cancer (Belinsky et al., 1998). The RAS effector homolog (*RASSF1*) gene has a putative role as a tumor-suppressor gene in lung cancer; *RASSF1* shows promoter methylation at the CpG islands in 40% of NSCLC, and loss of gene expression (Burbee et al., 2001). Finally, promoter methylation also affects the retinoic acid receptor system, which plays an important role in cell differentiation and lung development. Retinoids can suppress carcinogenesis in preneoplastic bronchial lesions and their effects are mediated by nuclear receptors, i.e., the retinoic acid receptors (*RAR*α, *RAR*β, and *RAR*γ) and the retinoid X receptors (*RXR*α, *RXR*β, and

RXRγ). Several reports indicate that loss of *RAR*β expression, because of promoter hypermethylation, is associated with increased susceptibility to lung cancer. The *RAR*β gene promoter is hypermethylated in 41% of NSCLC and is almost always unmethylated in control normal samples (Virmani et al., 2001b). Loss of promoter methylation in cell lines by in vitro treatment with 5-aza-2′-deoxycytidine restored *RAR*β gene expression and cell growth (Virmani et al., 2001b).

D. Histone Deacetylation

Another mechanism of gene silencing is represented by histone deacetylation. At variance with hypermethylation of gene promoter regions, histone deacetylation modulates higher-order chromatin structure. The addition of an acetyl group on lysines is catalyzed by histone acetyltransferase, while histone deacetylases remove the acetyl groups. Steady-state histone acetylation is controlled by the balance of both enzymatic activities; hypoacetylated histones increase their positive charge, condense the chromatin, and prevent gene transcription. Conversely, hyperacetylated histones neutralize the electrostatic charge and de-condense chromatin, thus allowing gene transcription to proceed. Transcription repressors, such as pRB, are associated with histone deacetylases. Recent studies have revealed several enzyme isoforms in mammalian cells encoded by histone deacetylase (HDAC) genes: HDAC1, HDAC2, HDAC3, h-HDAC4, h-HDAC5, h-HDAC6, h-HDAC7, and HDAC8 (Hu et al., 2000). There is potential synergy between inhibition of DNA methylation and histone deacetylase activity in restoring silenced gene expression. Indeed, depsipeptide, an inhibitor of histone deacetylase, acts synergistically with 5-aza-2′-deoxycytidine, a hypomethylating agent inhibitor of DNA-MTase, in restoring the expression of *CDKN2A* ($p16^{INK4a}$) (Zhu et al., 2001a) and inducing apoptosis in lung cancer cells (Zhu et al., 2001b). Apoptosis represents a naturally occurring mechanism of cell number regulation by deletion rather than by inhibition of cell division; abnormally triggered apoptosis also occurs after treatment with drugs that induce irreversible cell damage, such as cytotoxic agents, and also after withdrawal of hormones or growth factors or treatment with selected cytokines (Sloviter, 2002). Transforming growth factor (TGF)-β strongly inhibits epithelial cell proliferation through interaction with the TGF-β type II receptor (TGF-βRII). Most NSCLC cell lines have lost the growth-inhibitory response to TGF-β because of the loss of TGF-βRII expression, which is dependent, at least in part, on histone deacetylation (Osada et al., 2001). Finally, in vitro treatment of cells with the demethylating agent 5-aza-2′-deoxycytidine and the histone deacetylase inhibitor trichostatin A induces the cells to express hTERT (human telomerase reverse transcriptase), suggesting a potential role for DNA methylation and/or histone deacetylation in negative regulation of hTERT (Devereux et al., 1999). Agents that inhibit histone deacetylase in vitro include hybrid polar compounds (Richon et al., 1998), phenylacetate and phenylbutyrate (Samid et al., 1997), and MS-27-275 (Saito et al., 1999). These agents induce terminal differentiation in vitro as well as cell cycle arrest and partial reversion of the malignant phenotype in a variety of neoplasms, including NSCLC. For these reasons, inhibitors of histone deacetylase have been developed and clinically tested. The administration of the investigational agent CI-994 induced a partial response lasting over 2 years in one patient with heavily pretreated adenocarcinoma of the lung and a stable disease in an additional subject. Thrombocytopenia was the dose-limiting toxicity at the maximum tolerated dose of 8 mg/m^2/day for 8 weeks. Other toxicities included fatigue and gastrointestinal effects such as nausea, vomiting, diarrhea, constipation, and mucositis (Prakash et al., 2001).

E. Loss of Heterozygosity

Deletions of specific genes may occur during the development of tumors. This mechanisms of tumorigenesis, called loss of heterozygosity (LOH) or allelic imbalance, consists of the loss of an allele at a specific locus, and it is of obvious importance if this deletion involves a tumor-suppressor gene. Since the deletion involving only one allele may be silent, a second somatic mutation may consist of the loss of the entire chromosome, carrying the residual normal allele, or a large portion of it, or in the elimination of the normal gene by recombination events that duplicate the mutant allele (Black, 1997). In situations in which these events can be traced at the DNA level, for example by monitoring restriction fragment length or a cytidine-adenine repeat-type of polymorphism, the outcome is that the tumor appears to be homozygous or hemizygous for markers in or close to the relevant tumor-suppressor gene. The LOH is part of the *two-hit* model of carcinogenesis (Knudson, 1971; Black, 1997). Cells containing a pair of chromosomes in which a marker gene (e.g., retinoblastoma [*RB*]) is either homozygous wild-type or heterozygous have the same normal phenotype, demonstrating the recessive nature of the mutant allele. Tumorigenesis will only ensue if both copies of the gene are mutated (nonfunctioning) or deleted. In an individual who inherits a mutant copy from one parent, only a single somatic mutation is needed to lead to tumorigenesis. In subjects carrying two wild-type alleles, both copies must sustain independent somatic mutations (Black, 1997). The investigation of clinical implications of allelic deletions at three common sites of LOH in regions 5q21, 11p15.5, and 11p13 in 86 patients with NSCLC demonstrated that LOH frequency at 5q21 was 20%, whereas LOH frequencies in 11p15.5 and 11p13 were 31% and 19%, respectively (Sanchez-Cespedes et al., 1997). There was a significant correlation between 5q21 LOH and mediastinal lymph node involvement ($P = 0.03$); however, no significant differ-

ences were observed in median survival times in patients with 5q21 LOH as compared to the remainder (26 versus 37 months, $P = 0.33$) or in patients with 11p LOH (38 versus 32 months, $P = 0.72$) (Sanchez-Cespedes et al., 1997).

LOH for a locus on human chromosome 11q22–23 containing a putative tumor-suppressor gene is observed at high frequency in patients with NSCLC (Pletcher et al., 2001). LOH at the adenomatous polyposis coli/mutated in colonic cancer (APC/MCC) locus, a tumor-suppressor gene associated with both familial and sporadic cancer, was observed in 83% of NSCLC cell lines (Virmani et al., 2001a). It has been previously reported that the incidence of LOH on chromosomes 2q, 9p, 18q, and 22q in advanced-stage NSCLC was significantly higher than that in early stages (Shiseki et al., 1994, 1996). These results indicate that tumor-suppressor genes on chromosomes 2q, 9p, 18q, and 22q play an important role in the acquisition of malignant phenotype in NSCLC. However, the clinical implications and prognostic impact of 2q, 9p, 18q, and 22q LOH have not been established.

Transfer of chromosome 11 into the human A549 NSCLC cell line suppresses tumorigenesis, indicating that LOH may be responsible, at least in part, for the malignant phenotype and suggesting that multiple tumor-suppressor genes are located in this chromosome. A region of 700 kb on 11q23.2 of A549 cells also contains a single gene, *TSLC1* (tumor-suppressor lung cancer 1), whose expression is reduced or absent in A549 and several other NSCLC cell lines (Kuramochi et al., 2001). Hypermethylation of the TSLC1 promoter would represent the *second hit* in NSCLC with LOH (Kuramochi et al., 2001). A highly significant association between *TP53* mutations and deletions on 3p, 5q, 9p, 11p, and 17p is found in lung cancer (Zienolddiny et al., 2001). Furthermore, 86% of the tumors with concordant deletions in the four most involved loci (3p21, 5q11–13, 9p21, and 17p13) had *TP53* mutations as compared to only 8% of the tumors without deletions at the corresponding loci (Zienolddiny et al., 2001). The frequency of deletions was significantly higher among smokers as compared to nonsmokers. This difference was significant for the 3p21.3 (human MutL homolog-1 [hMLH1] locus), 3p14.2 (fragile histidine triad [FHIT] locus), 5q11–13 (human MutS homolog-3 [hMSH3] locus), and 9p21 (D9S157 locus). Deletions were more common in squamous cell carcinomas than in adenocarcinomas. Covariate analysis revealed that histological type and *TP53* mutations were significant and independent parameters for predicting LOH status at several loci (Zienolddiny et al., 2001). In a study designed to identify the major tumor-suppressor gene loci involved in the pathogenesis of lung cancer, 22 different regions with more than 60% LOH were identified: 1) 13 regions with a preference for SCLC, 2) 7 regions with a preference for NSCLC, 3) 2 regions affecting both SCLC and NSCLC (Girard et al., 2000). The chromosomal arms with the most frequent LOH were 1p, 3p, 4p, 4q, 5q, 8p, 9p (p16), 9q, 10p, 10q, 13q (*RB*), 15q, 17p (*TP53*), 18q, 19p, Xp, and Xq (Girard et al., 2000). In addition, new homozygous deletions were found at 2p23, 8q24, 18q11, and Xq22. On average, 36% of markers showed allele loss in individual NSCLC tumors, with an average size of subchromosomal region of loss of five to six markers. SCLC and NSCLC had different regions of frequent LOH (hot spots), and NSCLC had more of these regions ($n = 22$) than SCLC ($n = 17$) (Girard et al., 2000). Finally, in lung cancer cell lines, at least 17 to 22 chromosomal regions with frequent allele loss are involved, suggesting that the same number of putative tumor-suppressor genes is inactivated. In addition to this, SCLC and NSCLC frequently undergo different specific genetic alterations, and clusters of tumor-suppressor genes are likely to be inactivated together (Girard et al., 2000). Lung metaplastic and alveolar hyperplastic lesions with atypia show genetic alterations, including LOH of 3p, 9p, and mutations of the *TP53* gene. The analysis of microsatellite markers showed that 5 of 35 cases of squamous cell carcinoma and 3 of 26 cases of adenocarcinoma showed LOH in both preneoplastic lesions and synchronous cancers (Kohno et al., 1999). Nine patients (25.7%) with squamous cell carcinoma and 6 patients (23.1%) with adenocarcinoma had mutations involving *TP53*; in 2 patients with squamous cell carcinoma, the same mutation was observed in both dysplasia and squamous cell carcinoma (Kohno et al., 1999). These findings suggest that several genetic alterations may occur in preneoplastic lesions or in the early stage of squamous cell carcinoma of the lung, whereas they occur relatively late in the pathogenesis of adenocarcinoma (Kohno et al., 1999).

The analysis of surgically resected NSCLC specimens for LOH at 3p25–26, 3p21, 3p14, 5q, 11p, 17q, and 18q demonstrated that, with respect to pRB, $p16^{INK4a}$, and p53, the tumors could be grouped into four categories: normal for all three proteins (21%); abnormal for pRB or $p16^{INK4a}$ and normal for p53 (30%); normal for pRB and $p16^{INK4a}$ and abnormal for p53 (20%); and abnormal for all three proteins (28%) (Geradts et al., 1999). An aberrant expression of pRB, $p16^{INK4a}$, p53, and 3p LOH, either individually or in combination, was not associated with survival differences or any other clinical parameters, with the exception that pRB and $p16^{INK4a}$ abnormalities were more common in older patients. pRB and $p16^{INK4a}$ expression showed a strong inverse correlation, whereas there was no relationship between the expression of pRB, $p16^{INK4a}$, and p53 (Geradts et al., 1999). An abnormal expression of any of the three genes inversely correlated with *K-ras* mutations at codon 12 ($P = 0.004$), but not with LOH at 3p or at other loci. Therefore, NSCLCs show distinct patterns of tumor-suppressor gene inactivation, but no clear clinical correlates exist either alone or in combination for pRB,

$p16^{INK4a}$, p53, and 3p abnormalities (Geradts et al., 1999).

In an effort to identify regions containing novel cancer genes, chromosome 18p11 was examined for LOH in matched normal and NSCLC tumor samples by using 18p11 and 18q12.3 polymorphic markers (Tran et al., 1998). This analysis revealed two regions of LOH in 18p11 in up to 38% of the tumor samples examined. The regions of LOH identified included a region between D18S59 and D18S476 markers, and a more proximal region of intermediate frequency between D18S452 and D18S453 (Tran et al., 1998). These results provide evidence for the presence of one or more tumor-suppressor genes on the short arm of chromosome 18, which may be involved in NSCLC (Tran et al., 1998). Deletions in the 5q14 region have been described in a variety of neoplasms, including lung cancer. The high frequency of allelic losses observed in this region implies the presence of putative tumor-suppressor genes. In a series of 56 NSCLCs the allelic imbalance within the 5q14 region and its relationship with p53 abnormalities, kinetic parameters, proliferation and apoptotic index, and the ploidy status of tumors revealed that an allelic imbalance at D5S644 was found at a frequency of 51.2% (Gorgoulis et al., 2000). LOH at 5q14 was associated with a low apoptotic index, suggesting the presence of putative tumor-suppressor genes. Simultaneous alterations of both p53 and D5S644 loci were the most frequent pattern observed (37.5%) (Gorgoulis et al., 2000). These findings imply a synergistic mechanism of cooperation between different tumor-suppressor genes. However, proliferation activity was dependent only on p53 status, leading to the assumption that the putative tumor-suppressor genes present at 5q14 may be involved in apoptotic pathways (Gorgoulis et al., 2000). The use of microsatellite markers at 3p14, 9p21, and 10q24 to analyze tumor samples from 91 patients with pathological stage I NSCLC demonstrated that LOH at any single locus was not significantly associated with survival (Zhou et al., 2000). The analysis of LOH on a panel of 102 NSCLC samples with 20 polymorphic markers evidenced two short regions of the overlap of the deletions (SROs): SRO2a (D1S417-D1S57) and SRO2b (D1S450-D1S243). Allelic losses at either region located on 1p32-pter correlated independently with an advanced stage of disease and with postoperative metastasis and relapse, suggesting that crucial genes in these regions are involved in NSCLC progression (Chizhikov et al., 2001). The short arm of chromosome 3 is thought to harbor an oncogenic locus that is important in lung carcinogenesis because of its sensitivity to loss by the action of carcinogens and evidence of frequent deletion in lung cancer. Of 219 lung cancers, 44.2% of squamous cell carcinomas and 30.2% of adenocarcinomas showed 3p21 LOH, its prevalence being higher in p53 mutated cases (Hirao et al., 2001). The analysis for LOH at chromosome 3p24 in samples of normal and tumor tissues from the lungs of 76 patients with NSCLC revealed that *RXRβ*, *RARβ*, and *RARγ* gene expression was decreased in 18%, 63%, and 41% of tumor specimens (Picard et al., 1999). LOH at 3p24 was observed in 41% of tumor samples and in 20% of non-neoplastic lesions. Therefore, a large percentage of tumors shows a marked decrease in the expression of *RXRβ*, *RARβ*, and *RARγ*, and a high frequency of LOH at 3p24, which is also observed in non-neoplastic lesions (Picard et al., 1999). These data suggest that altered retinoid receptor expression may play a role in lung carcinogenesis (Picard et al., 1999). LOH at chromosome 3p24, which hosts *RARβ*, was observed in 100% (13 of 13) of SCLC cell lines and 67% (12 of 18) of NSCLC cell lines, and the difference was statistically significant (Virmani et al., 2001b). Abnormalities of FHIT, the tumor-suppressor gene located at 3p14.2, have been found in NSCLC. Analysis of a subset of 76 specimens of stage I NSCLC, in which microsatellite analysis at the *FHIT* locus was performed, did not show a strong association between LOH at 3p14.2 and pFHIT expression, suggesting the presence of complex mechanisms of gene inactivation (Tseng et al., 1999). However, loss of *FHIT* was significantly higher in bronchial metaplastic lesions (47%) than in histologically normal bronchial epithelium (20%), and pFHIT expression was significantly reduced in a substantial number of early-stage NSCLC and preneoplastic lesions in chronic smokers (Tseng et al., 1999).

In stage I NSCLC, allelic imbalance is observed on 2q, 9p, 18q, and 22q in 22, 38, 29, and 15% of cases, respectively, whereas p53 is mutated in 41% of stage I NSCLCs (Tomizawa et al., 1999). Allelic imbalance on 9p and 22q, and p53 mutations, were significantly associated with shortened survival of the patients (Tomizawa et al., 1999). These results indicate that clinical aggressiveness of early-stage NSCLC is associated, at least in part, with the presence of allelic imbalance on chromosome 9p, which could be a clinically useful prognostic indicator (Tomizawa et al., 1999). Allelotyping studies suggest that allelic losses at one or both arms of chromosome 4 are frequent in several tumor types. The analysis of clinical specimens and NSCLC cell lines by using 16 polymorphic microsatellite markers showed LOH at three nonoverlapping regions: 1) 4q33–34 (R1), 2) 4q25–26 (R2), and 3) 4p15.1–15.3 (R3) in about 20 to 30%, with no differences between tumors and cell lines, the loss of R3 alone being the most frequent pattern (Shivapurkar et al., 1999). LOH may occur in 83% of NSCLCs with chromosomal duplication, suggesting that the duplicated chromosome is homozygous; these findings imply that LOH occurs before chromosomal duplication during lung carcinogenesis (Varella-Garcia et al., 1998). LOH on chromosome 11q23 is observed at high frequency in NSCLC, suggesting the presence of a tumor-suppressor gene (Murakami et al., 1998). Allelotyping of NSCLC and SCLC cell lines demonstrated significant differences in LOH frequencies between NSCLC

and SCLC at 13 regions on 8 chromosome arms (3p, 5q, 6q, 9p, 10q, 11p, 13q, and 19p). Eight homozygous deletions were present in seven cell lines at four regions, 3p12, 3p14.2, 9p21, and 10q23–25. In addition to this, there was LOH at 6p21.3 and 13q12.3 in NSCLC (Virmani et al., 1998). The frequent occurrence of 21q deletions in human NSCLC indicates the presence of a tumor-suppressor gene on this chromosome arm. The *ANA* (abundant in neuroepithelium area) gene, a member of an antiproliferative gene family, is mapped to 21q11.2-q21.1 and was homozygously deleted in the human Ma17 NSCLC cell line. LOH at this locus was detected in 24 of 47 (51.1%) NSCLCs, and the frequency of LOH in brain metastases was significantly higher than that in stage I–II primary tumors. These data suggest that the homozygously deleted region harbors a novel tumor-suppressor gene involved in NSCLC progression (Kohno et al., 1998). The *PTEN/MMAC1* (phosphatase and tensin homolog deleted on chromosome 10/mutated in multiple advanced cancers) is a candidate tumor-suppressor gene recently identified at chromosomal band 10q23. Microsatellite analysis revealed LOH at markers near the gene in 50% of 42 primary NSCLCs. These results suggest that *PTEN/MMAC1* gene inactivation plays a role in the genesis of some tumor types (Okami et al., 1998). In a cohort of 87 NSCLCs, LOH was investigated by using dinucleotide repeat sequences from chromosomal locations 1p, 3p, 5q, 8p, 9p, 10p, 11p, 13q, and 17q. In 28% (24 of 87) of NSCLCs, LOH in at least one locus was detected. The frequency of LOH differed between the various cell types of NSCLC. The highest frequency was seen in large cell carcinoma (3 of 6, 50%) followed by squamous cell carcinoma (16 of 43, 37%) and adenocarcinoma (5 of 35, 14%), and the most common site of LOH was 3p (Pylkkanen et al., 1997). The *TGF-βRII* gene has been mapped to chromosome 3p, on which LOH was frequently detected in NSCLC; however, *TGF-βRII* mutations were not found in NSCLC with LOH on chromosome 3p (Tani et al., 1997). Deletions involving the chromosome 9p21 region, which also harbors the tumor-suppressor locus *CDKN2A*, have been reported as frequent events in NSCLC. LOH at a marker proximal to the *CDKN2A* locus was found most frequently (52%), while LOH at a marker closest (5 kb) to the *CDKN2A* gene was seen in only 17% of tumors (Mead et al., 1997). A homozygous loss of markers close to *CDKN2A* was, however, detected in 2 of 3 cell lines and one accompanying tumor sample. Therefore, a tumor-suppressor gene in the region of deletion proximal to the *CDKN2A* gene within 9p21 may play a significant role in the pathogenesis and progression of NSCLC (Mead et al., 1997). LOH in the *TP53* locus was found in 9 of 38 (23.6%) cases. A trend was found between p53-positive immunostaining and a history of heavy smoking, and was inversely correlated with LOH at the *TP53* locus (Liloglou et al., 1997). High LOH on chromosome arms 3p, 9p, and 17p is a common event in NSCLC. LOH was observed at a frequency of 38% on 3p, 58% on 9p, and 38% on 17p. Polarization of the LOH on chromosome arms 3p, 9p, and 17p was observed such that 80% showed loss on 3p, 80% on 9p, and 73% on 17p (Field et al., 1996). LOH on chromosome arms 3p, 13q, and 17p was detected frequently (>60%) in both stage I primary lung tumors and brain metastases, whereas the incidence of LOH on chromosome arms 2q, 5q, 9p, 12q, 18q, and 22q was higher than 60% only in brain metastases. In particular, the incidence of LOH on chromosome arms 2q, 9p, 18q, and 22q in brain metastases was significantly higher than that in stage I primary lung tumors (Shiseki et al., 1996). These results indicate that tumor-suppressor genes on chromosome arms 3p, 13q, and 17p are involved in the genesis of NSCLC, whereas those on several chromosome arms, especially on 2q, 9p, 18q, and 22q, play an important role in the progression of NSCLC (Shiseki et al., 1996). High-density polymorphic marker analysis throughout 11p15.5 confirmed the presence of two distinct regions of LOH for NSCLC in 11p15.5. In 9 of 13 (69%) tumors with LOH, allelic deletion was restricted to 11p15.5, indicating that whole chromosome 11 loss is not a common event in NSCLC and suggesting that chromosome band 11p15.5 harbors a minimum of three separate loci (Tran and Newsham, 1996).

3p21 Loss appears, so far, to be the most frequent and the earliest genetic alteration described in NSCLC, but it does not seem to carry significant prognostic information in invasive tumors (Thiberville et al., 1995). The short arm of chromosome 17, which contains the p53 gene, is frequently affected by LOH in lung cancer. The frequency of LOH at 17q is 42%, approaching that at 17p (54%), and two distinct 17q regions are implicated. LOH at D17S4 on 17q is more frequent in adenocarcinomas than in squamous cell carcinomas, whereas squamous cell carcinomas had more LOH at 17p than at 17q, indicating a molecular genetic heterogeneity between the major NSCLC subtypes. In addition, LOH at 17q correlates with higher tumor stages and a significantly worse prognosis. In comparison, 25% of cases have mutations at *TP53* exons 5–8, but these are not associated with tumor stage or survival (Fong et al., 1995a). LOH at the *APC/MCC* gene cluster at chromosome 5q21 occurs frequently in NSCLC; it affects 29% of NSCLC and it is significantly correlated with worse survival. Furthermore, in squamous cell carcinoma, LOH at 5q not only correlated with a short survival, but also with tumor involvement of the mediastinal and/or hilar lymph nodes (Fong et al., 1995b). In contrast, LOH at chromosome 18q was far less frequent, occurring in 14% of NSCLC cases, and it was not associated with advanced stage or adverse prognosis. These data suggest that LOH at 5q has a role in determining tumor progression and survival in NSCLC, and may prove to be a clinically useful prognostic indicator (Fong et al., 1995b).

F. Microsatellite Alteration

Microsatellites are repetitive nucleotide sequences of varying lengths, which occur in the human genome, between and within genes (Eshleman et al., 1996; Sherbet and Lakshmi, 1997). Microsatellite sequences (also called *microsatellite markers*) are unstable because of variations that can occur in repetitive sequence units, resulting in the expansion or shortening of them. The instability of microsatellite loci contributes to the *mutator phenotype* of cancer and provides an explanation of the high incidence of mutations compared to normal cells (Loeb, 1994). The instability of microsatellites can affect nonrepetitive sequences of the DNA, and the direct consequence is the generation of a ladder-like motif that replaces the normal allele pattern of the human genome (Wistuba et al., 2001). A majority of microsatellite repeats occur outside the coding regions of genes; therefore, microsatellite instability may not directly lead to carcinogenesis, but could destabilize DNA sequences inside and outside the microsatellite repeats and make the genome hypermutable. As a corollary, one should consider the possibility that microsatellite instability might be engendered by exposure to carcinogens. Microsatellite instability was found in about one-third of NSCLC, with a substantial difference between metastatic lesions (55%) and primary disease (12%) (Adachi et al., 1995), thus suggesting a possible direct relationship between microsatellite instability and cancer progression. It has been recently observed, however, that microsatellite instability, defined as the change in the number of short-tandem DNA repeats, is not common in NSCLC, while the *microsatellite alteration*, where a single band of altered size is found, has been described in 2 to 49% of NSCLC (Sekido et al., 1998; Wistuba et al., 2001). By using 16 markers on chromosomes 3p and 9p, microsatellite alteration is found in 7 of 20 histologically normal lung tissue specimens at a frequency similar to that observed in NSCLC tumor tissue (8 of 20). Five cases showed microsatellite alteration in both normal lung tissue and the corresponding tumor (Park et al., 2000). In 2 of 12 patients microsatellite alteration was detected in normal lung tissue while the tumor was negative. These results indicate that genetic alterations are widely distributed in the lung tissue of patients with lung cancer (Park et al., 2000).

The short arm of chromosome 3 is thought to harbor an oncogenic locus involved in the pathogenesis of NSCLC. The region at 3p21 is believed to contain a distinct locus that is sensitive to loss from the action of tobacco smoke carcinogens, and has been reported to be specifically targeted for deletion in lung cancer. A recent study examined the LOH on chromosome 3 at 3p21 in NSCLC and the microsatellite alteration at the BAT-26 locus because the mismatch DNA repair gene, hMLH1, is found at 3p21 (Hirao et al., 2001). Instability of BAT-26 was not found, while LOH at 3p21 was detected in 44.2% of squamous cell carcinomas and 30.2% of adenocarcinomas and was frequently associated with *TP53* mutations (Hirao et al., 2001). Using a panel of 12 markers, microsatellite instability was detected in 24 of 47 (51%) NSCLC and 10 of 18 (56%) head and neck cancers, but was only observed in 8 of 38 (21%) bladder and 3 of 25 (12%) kidney cancers (Xu et al., 2001). The results of this study suggest that about 50% of respiratory tract cancers exhibit microsatellite instability, predominantly at AAAG sequences. This distinct type of instability is termed EMAST (elevated microsatellite alterations at selected tetranucleotide repeats) and the identification of markers with EMAST may prove useful for the molecular detection of respiratory tract cancers (Xu et al., 2001). Microsatellite instability was observed in 5 of 7 NSCLC cell lines and 3 of 21 NSCLC tissues (Kim et al., 2000). Microsatellite instability was highly associated with *TGF-βRII* frameshift mutations (75%), thus supporting the hypothesis that *TGF-βRII* plays an important role in NSCLC carcinogenesis (Kim et al., 2000). Among 91 patients with stage I NSCLC, 32% of subjects whose tumors had microsatellite instability at 10q24 died of the disease within 5 years after surgery, compared with 16% without microsatellite instability at 10q24 (Zhou et al., 2000). Seventy-one percent of patients with lung adenocarcinoma and microsatellite instability at 10q24 died because of disease progression, compared with 12% without microsatellite instability, indicating the presence of distinct mechanisms in tumorigenesis among different subtypes of lung cancer (Zhou et al., 2000). Of 23 patients who had microsatellite instability at 10q24 and 3p14, 39% died of the disease within 5 years as compared with 15% of the patients without such a profile (Zhou et al., 2000). Furthermore, among the 22 patients with no alteration at any loci tested, none died of lung cancer within 5 years after surgery, whereas 28% of the patients outside these profiles died of the disease (Zhou et al., 2000). These results support the hypothesis that microsatellite alterations can be used as biomarkers for the genetic classification of stage I NSCLC, which may in turn influence treatment decisions (Zhou et al., 2000). Microsatellite alteration may also be detected in the DNA of cells in bronchoalveolar lavage fluid from patients with resectable NSCLC; indeed, microsatellite instability was observed in NSCLC tissue in approximately 50% of patients; the identical alteration was shown in the bronchoalveolar lavage fluid of 14% of the corresponding patients (Ahrendt et al., 1999).

Chromosome 3p is consistently deleted in lung cancer, and it is believed to contain several tumor-suppressor genes. The role of chromosome 3 in tumor suppression has been confirmed by isolation of the human homolog of the ribosomal protein L14 gene (RPL14) located at 3p21.3 (Shriver et al., 1998). The RPL14 sequence contains a highly polymorphic trinucleotide repeat that encodes a variable-length polyalanine tract (Shriver et al.,

1998). Genotype analysis of RPL14 shows that this locus is 68% heterozygous in the normal population compared with 25% in NSCLC cell lines. Cell cultures derived from normal bronchial epithelium show a 65% level of heterozygosity, reflecting that of the normal population (Shriver et al., 1998). In additional studies, microsatellite instability at one or more loci was observed in 13 (36%) of 36 cases of resected NSCLC (19 cases of squamous cell carcinoma, 15 of adenocarcinoma, and 2 of large cell carcinoma) (Kim et al., 1998a). Six tumors showed instability in a single microsatellite, three tumors had alterations in three of four tested microsatellites, and the microsatellite that showed instability most frequently in these tumors was D3S1340 (31%) (Kim et al., 1998a). Furthermore, microsatellite instability was found in 24% of 17 cancers at stage I, in 17% of 6 tumors at stage II, in 73% of 11 tumors at stage IIIA, and in none at stage IIIB; overall, microsatellite instability was observed in at least one-third of NSCLC (Kim et al., 1998a). A set of 11 microsatellite loci spanning 1p was used to examine the frequency of allelic imbalance in a panel of 58 tumors; 87.9% of 58 cases had somatic allelic loss at one or more loci tested. Two SROs have been identified: SRO1 at 1p13.1 and SRO2 at 1p32-pter (Gasparian et al., 1998). Allelic losses at these regions have been compared among adenocarcinomas and squamous cell carcinomas, and no difference has been found. On the contrary, SRO2 deletions significantly correlated with advanced stage of the disease and postoperative disease recurrence (Gasparian et al., 1998). These data may suggest that SRO1 and SRO2 harbor tumor-suppressor genes involved in different stages of NSCLC development (Gasparian et al., 1998). The comparison of DNA from human tumor and normal bronchial mucosa with respect to microsatellite instability and LOH on chromosome 17p, 17q, 9p, and 9q, using 10 polymorphic markers, was performed on biopsies and tissue specimens obtained from the tumor and paired normal bronchial mucosa in 20 patients with NSCLC (Froudarakis et al., 1998). Sixteen of 20 tumors (80%) displayed genetic alterations; 30% of tumors exhibited microsatellite instability, 25% exhibited LOH, and 25% of tumors showed microsatellite instability and LOH (Froudarakis et al., 1998). No relationship was found between LOH or microsatellite instability and the histologic subtype of NSCLC or disease stage. These results suggest that genetic alterations have a role in carcinogenesis as they exist in all stages and histologic subtypes of NSCLC (Froudarakis et al., 1998). In a cohort of 379 women with NSCLC, microsatellite instability was observed more frequently in patients with three or more relatives with cancer (6 of 9, 67%) than in control patients (5 of 28, 18%; $P = 0.011$) (Suzuki et al., 1998). Thus, a significantly higher rate of microsatellite instability is associated with familial clustering of malignancy (Suzuki et al., 1998). The replication-error-type instability (RER+) is a frequent genetic alteration in stage I NSCLC. RER+ at one or both chromosomes 2p and 3p was identified in 24 of 35 patients; 9 patients showed LOH (Rosell et al., 1997). A statistically significant correlation was found between RER+ and poor prognosis; furthermore, RER+ proved to be an independent factor that predicted decreased survival (Rosell et al., 1997). These data suggest that RER+ is common in NSCLC, and it may provide important prognostic information in stage I NSCLC (Rosell et al., 1997).

G. Protein Phosphorylation

Reversible protein phosphorylation has emerged as the predominant mechanism of control of protein activity in eukaryotic cells in response to environmental signals, mainly related to cell proliferation. The phosphorylation of specific proteins, which is under the control of two families of enzymes known as protein kinases and phosphatases, provides signal amplification. Since more than 10% of proteins in a normal mammalian cell are thought to be regulated through phosphorylation, this aspect of proteomics is gaining significant interest. Abnormal protein phosphorylation is the basis for or the result of major diseases, including cancer. Mutations in protein kinases and phosphatases or in regulatory genes result in a number of hereditary disorders, including leukemias and lymphomas (Shapiro et al., 1995). Excessive activity of kinases under the control of growth-promoting genes is the apparent mechanism responsible for inactivation of tumor-suppressor gene products, including pRB. Indeed, cyclin-dependent kinase (cdk)4-mediated phosphorylation of pRB is stimulated by cyclin D1, an oncogene, and inhibited by $p16^{INK4a}$, the product of the tumor-suppressor gene *CDKN2A* (Shapiro et al., 1995). NSCLC is predominantly pRB-positive and most tumor specimens and cell lines overexpress cyclin D1, indicating that cyclin D1 overexpression and *RB* inactivation coexist (Shapiro et al., 1995). Furthermore, pRB-positive NSCLC cell lines have absent or low $p16^{INK4a}$, and in primary lung resection specimens $p16^{INK4a}$ was undetectable in 18 of 27 NSCLC samples. These data confirm the dependence of pRB inactivation on $p16^{INK4a}$ expression (Shapiro et al., 1995).

To evaluate the role of Akt/PKB (AKR mouse T-cell lymphoma/protein kinase B) in the survival of patients with NSCLC, a panel of NSCLC cell lines that differed with respect to tumor histology and p53, pRB, and p21K-ras status were examined. Constitutive Akt/PKB activity was demonstrated in 16 of 17 cell lines (Brognard et al., 2001). Akt/PKB activation was dependent on phosphatidylinositol 3-kinase (PI3K) and promoted survival because wortmannin, a PI3K inhibitor, suppressed Akt/PKB phosphorylation and increased apoptosis only in cells with activated Akt/PKB (Brognard et al., 2001). To test whether Akt/PKB is involved in drug resistance, tumor cells were exposed to conventional anticancer agents in combination with the phosphatidyl-inositol 3-kinase inhibitor LY294002. LY294002 potentiated

chemotherapy-induced apoptosis in cells with high Akt/PKB levels, but was ineffective in cells with low Akt/PKB levels (Brognard et al., 2001). Transfecting constitutively active Akt/PKB into cells with low Akt/PKB activity attenuated chemotherapy- and radiation-induced apoptosis (Brognard et al., 2001). Thus, Akt/PKB is a constitutively active kinase that promotes survival of NSCLC cells, and modulation of its activity by pharmacological or genetic approaches alters the cellular sensitivity to chemotherapeutic agents used to treat patients with NSCLC (Brognard et al., 2001).

IV. Genetic Abnormalities in Non-Small Cell Lung Cancer

Multistep tumorigenesis is the process by which genetic events accumulate over time and result in malignant transformation. It is estimated that approximately 10 to 20 alterations of tumor-suppressor genes and/or proto-oncogenes are required for lung tumorigenesis. Numerous alterations have been identified that occur frequently in NSCLC. These include *RAS* proto-oncogene mutations, *TP53* gene mutations, inactivation of the *RB* gene, and alterations in *CDKN2A*, *HER-2/neu*, *MYC*, *Bcl-2*, and *FHIT* (Table 2).

A. RAS

The product of the *RAS* gene (p21ras) regulates transduction of growth-proliferative signals from the membrane to the nucleus, and mutationally activated *RAS* is found in 25 to 48% of NSCLC. The p21ras proteins bind to and hydrolyze GTP by means of their intrinsic GTPase activity. Point mutations in p21ras impair its GTPase activity and the constitutive presence of the active, GTP-bound form of p21ras ($p21ras^{GTP}$) leads to deregulated growth and cellular transformation (MacDonald and McCormick, 1997). To perform its function in cell signaling, p21ras must be farnesylated on the CAAX motif (*C*ysteine, *A*liphatic amino acid, and any amino acid [*X*]) at the carboxyl terminus of p21ras protein, a reaction mediated by farnesyl protein transferase (Di Paolo et al., 2001). Intracellular effectors of p21ras include raf-1, MEK (mitogen-activated protein kinase [MAPK]/extracellular signal-regulated kinase [ERK] kinase), and MAPKs, which are needed for *RAS*-mediated DNA synthesis, gene transcription, and eventually malignant transformation (MacDonald and McCormick, 1997). Although this represents the prevalent hypothesis for p21ras signal transduction, recent studies failed to substantiate it (Ramakrishna et al., 2000). In particular, lung tumors do not have more total p21K-ras or $p21K\text{-}ras^{GTP}$ than normal lung tissue, nor are higher levels of these proteins found in tumors with mutant *K-RAS*. Activated $p21K\text{-}ras^{GTP}$ levels did not correlate with proliferating cell nuclear antigen (PCNA) staining. Furthermore, tumors with mutant *K-RAS* displayed smaller size compared with tumors lacking this mutation (Ramakrishna et al., 2000). In nontransformed lung epithelial cells in culture both total and activated p21K-ras increased markedly at confluence, but not after serum stimulation, and mRNA analysis indicated an increase in *K-RAS* expression in confluent cells. These findings indicate that normal p21K-ras activity is associated with growth arrest of normal lung epithelial cells and that the exact contribution of mutated p21K-ras to tumor development is still undetermined (Ramakrishna et al., 2000). To evaluate the association of *K-RAS* abnormalities with the incidence of NSCLC, 410 surgically resected specimens were analyzed for *K-RAS* mutations in codons 12, 13, and 61; mutations were found in 33 patients (8%) and all were smokers or ex-smokers (Noda et al., 2001). There were no significant differences in tumor stage between wild-type and mutant *K-RAS*. The most frequently identified mutation was a G>T transversion (75.8%) that resulted in the substitution of a glycine for a cysteine or a valine (Noda et al., 2001). This study provides evidence of a clear correlation between smoking and G>T transversions affecting the *K-RAS* gene (Noda et al., 2001). Survival is strongly associated with *K-RAS* gene mutations in NSCLC (Rosell et al., 1996). The analysis of the relationship between tumor aggressiveness and *K-RAS* point mutations at codons 12 and 61 was evaluated in 275 consecutively treated stage I–IV NSCLCs. In stage I disease, median survival was 27 versus 41.5 months in patients with or without *K-RAS* mutations at codon 12, respectively (Rosell et al., 1996). Furthermore, in patients with stage IIIA disease, median survival time was 7 months in those with *K-RAS* mutations at codon 12 (aspartic acid to serine) and 15 months for those with other *K-RAS* mutations ($P = 0.01$) (Rosell et al., 1996). In a multivariate analysis, point mutation at codon 12 of *K-RAS* was a strong predictive factor for death (hazard ratio, 2.06; $P = 0.02$) after adjustment for other factors, including stage and histology. Therefore, in patients with NSCLC specific *K-RAS* point mutations are associated with a significantly increased risk of recurrence and death, independently of tumor stage and histology (Rosell et al., 1996).

Intron 1 of the human *H-RAS* gene possesses a unique polymorphism consisting of GGGCCT repeats. Analysis of this locus in matched tumor versus normal samples from 38 patients with NSCLC revealed 6.6% LOH and 10.5% hexanucleotide instability (Kotsinas et al., 2001).

TABLE 2
Summary of genes involved in lung tumorigenesis

Gene	Protein Function	Chromosomal Location
K-RAS	Signal transduction	11p14.1
TP53	DNA repair checkpoint	17p13
RB	Cell cycle checkpoint	13q14
CDKN2A	CDK inhibitor	9p21
MYC	Transcription factor	8q24
Bcl-2	Inhibitor of apoptosis	18q21
FHIT	Tumor suppressor	3p14.2
HER-2/neu	Signal transduction	17p21

The same pattern of alterations was also detected in tissues adjacent to lung adenocarcinomas and dysplasias contiguous to squamous cell carcinomas (7.7% LOH, 5.9% hexanucleotide instability), implying that abnormalities at this locus may be early events in lung carcinogenesis (Kotsinas et al., 2001). In view of reports showing that elements in intron 1 of the *H-RAS* gene potentially influence its transcriptional regulation, the hexanucleotide locus could be an element with possible involvement in expressional regulation of *H-RAS* (Kotsinas et al., 2001).

B. TP53

The product of the *TP53* tumor-suppressor gene is p53, a DNA-binding, sequence-specific transcription factor that activates the expression of genes engaged in promoting growth arrest in the G_1 phase or cell death in response to genotoxic stress. Also, p53 prevents cells from undergoing mitosis when they enter the G_2 phase with damaged DNA (Taylor and Stark, 2001). Part of the mechanism by which p53 blocks cells at the G_2 checkpoint involves inhibition of cdc2, the cyclin-dependent kinase required to enter mitosis. Binding of cdc2 to cyclin B1 is required for its activity, and repression of the cyclin B1 gene by p53 also contributes to blocking entry into mitosis (Taylor and Stark, 2001). The transfer of the wild-type *TP53* gene into the p53-null human NSCLC NCI-H358 cells results in a typical senescence-like phenotype, characterized by reduction in cell growth, enlarged and flat cell morphology, cell cycle arrest in the G_1 phase, down-regulation of cyclin B1 and cdc2 expression, and suppression of DNA synthesis (Ling et al., 2000). The ability of p53 to inhibit cellular proliferation or to induce cell death is suppressed by the product of the mouse double minute 2 (*MDM2*) gene. This property underlies the oncogenic potential of *MDM2*, which is overexpressed in various human tumors. Similar to other oncogenes, surveillance pathways might counteract the deleterious effects of deregulated *MDM2* expression (Daujat et al., 2001). The GML gene (glycosyl-phosphatidyl-inositol-anchored molecule-like protein gene) is a novel gene specifically induced by wild-type p53, which may participate in cell cycle control and the apoptotic pathway (Higashiyama et al., 2000). Loss of function of the *TP53* tumor-suppressor gene, because of missense mutations that cause single residue changes in the DNA binding core domain of the protein, occurs early in lung tumorigenesis in about 50% of cases (Rom et al., 2000). This mutation confers a growth advantage to the cells, allowing them to clonally expand due to loss of the p53-controlled G_1 checkpoint and apoptosis. Genetic instability due to the impaired ability of p53 to regulate DNA damage repair further facilitates the occurrence of new genetic abnormalities, leading to malignant progression. The cell cycle control is further compromised in NSCLC by alterations in the G_1/S transition control genes, either loss of the *CDKN2A* or *RB* genes or amplification of the cyclin D gene (Sherbet and Lakshmi, 1997). Not only *TP53* mutations result in the abrogation of wild-type p53 activity, but the expressed p53 mutant proteins also tend to gain oncogenic functions, such as interference with wild-type p53-independent apoptosis (Sigal and Rotter, 2000). Because mutated p53 is expressed in cancer cells and not in normal cells, its reactivation to wild-type function by gene transduction may eliminate cancer by restoring apoptosis or other p53-dependent mechanisms of growth suppression (Sigal and Rotter, 2000). However, mutants with more extensive structural changes in the DNA binding core domain may be refractory to reactivation to the wild-type p53 phenotype. Therefore, understanding the structure and functions of p53 mutants may lead to effective reactivation modalities or to the ability to eliminate mutant p53 (Sigal and Rotter, 2000).

The development of p53 gene abnormalities, which result in gross aneuploidy and multiple structural chromosomal changes, commonly occurs at the interface between severe dysplasia and invasive tumor (Shackney and Shankey, 1997). To date, the characteristics of *TP53* gene mutations in lung cancer have been extensively investigated. However, current estimates of *TP53* alterations are inaccurate, since most studies have limited their analyses to exons 5 to 8 of the *TP53* gene. The examination of mutations in the entire coding region of *TP53*, from exons 2 to 11, in 52 lung carcinoma cell lines and 106 primary NSCLCs revealed that the prevalence of mutations was high (>80%) in NSCLC cell lines, and 9 of 45 mutations (20%) were detected outside the region of exons 5 to 8 (Fujita et al., 1999). The frequency of mutations in primary NSCLC was 48% and was significantly different between adenocarcinomas (39%) and squamous cell carcinomas (67%). A>G transitions (14%) and G>T transversions (26%) were frequently detected in smoking patients (Fujita et al., 1999).

The DNA repair protein MGMT removes mutagenic adducts from the O^6 position of guanine, thereby protecting the genome against G>A transitions (Wolf et al., 2001). The *MGMT* gene is inactivated by promoter hypermethylation in many human cancers and has been associated with G>A mutations in *K-RAS*. In addition to this, experimental data have been provided in support of the hypothesis that hypermethylation of the *MGMT* promoter is associated with an increase in G>A transitions in the *TP53* gene in NSCLC (Wolf et al., 2001). Methylation of the promoter region of the *MGMT* gene is detected in 29% of NSCLC primary tumors collected at surgery; hypermethylation of the *MGMT* promoter was more common in adenocarcinoma than in other histological types of NSCLC and was also more common in poorly differentiated tumors (Wolf et al., 2001). *MGMT* promoter hypermethylation occurred significantly more often (64%) in tumors with a G>A mutation in *TP53* than in tumors with other types of *TP53* mutations (27%) or in tumors with wild-type *TP53* (18%) (Wolf et

al., 2001). The shift in the *TP53* mutational status observed in methylated tumors suggests that *MGMT* promoter hypermethylation precedes the mutation of the *TP53* gene. Because G>A mutations account for more than 40% of the *TP53* mutations in human cancer, it is likely that, in most cases, MGMT inactivation play a major role (Wolf et al., 2001).

C. RB

The *RB* tumor-suppressor gene is located on chromosome 13q14. Cytogenetic abnormalities of chromosome 13 and LOH at the *RB* locus have been reported in a variety of human cancers, including NSCLCs, and the frequency of *RB* abnormalities detected by immunohistochemistry in NSCLCs is up to 30% (Wistuba et al., 2001). Furthermore, functional pRB protein is absent in 90% of SCLCs, and in up to 30% of NSCLC primary lesions and cell lines (Rom et al., 2000). pRB is a pocket protein that cooperates with p53 in the regulation of cell cycle progression and controls, at the transcriptional level, the balance between cell differentiation and proliferation (Sherbet and Lakshmi, 1997). In the cell cycle, the transition from the G_1 to the S phase is of crucial importance. Indeed, it is only before this checkpoint that cells can be oriented toward the differentiation pathway; beyond, cells progress into the cycle in an autonomous manner. The transcription factor E2F controls the expression of a group of checkpoint genes whose products are required either for the G_1/S transition or DNA replication (e.g., DNA polymerase-α, thymidylate synthase, thymidine kinase, and dihydrofolate reductase). E2F activity is repressed in growth-arrested cells and in early G_1, and is activated at mid-to-late G_1. pRB represses E2F by binding to it and by activating chromatin remodeling factors, including histone deacetylases, DNA-MTase, and histone methyltransferase (Ferreira et al., 2001; Nevins, 2001). The molecular events that lead to deregulated tumor cell growth include sustained activity of cyclin-dependent kinases (CDK2, CDK4, and CDK6); as a result of loss of CDK inhibitors, such as $p16^{INK4a}$; and persistent up-regulation of several cyclins (cyclin D1, cyclin A, and cyclin E), the positive regulators of CDKs. CDKs inactivate pRB by phosphorylation, which is followed by the release of E2F from an inactive complex with pRB, and the constitutively high E2F activity induces continuous expression of target genes whose products promote cell cycle progression (La Thangue, 1997; Sherbet and Lakshmi, 1997). Therefore, sustained hyperphosphorylation and inactivation of pRB contribute to the transformation of normal bronchial epithelium to autonomously growing cancer cells. Additional mechanisms of pRB inactivation include *RB* point mutations and chromosomal deletions (La Thangue, 1997; Sherbet and Lakshmi, 1997). Although *RB* plays an important role in lung tumorigenesis, in cooperation with other genetic abnormalities pRB status does not represent a prognostic factor in NSCLCs. Indeed, among 90 patients with lung adenocarcinoma, 56.7% have reduced expression of the *RB* gene; however, there were no statistical differences among pRB, *TP53* mutations, and clinico-pathological status. The 5-year survival rate in patients with normal versus reduced pRB expression was 55.1 versus 73%, the difference being nonsignificant (Sugio et al., 2001). Furthermore, in tumor specimens obtained from 207 surgically resected primary NSCLCs, p53 or pRB were detected in 55.6 and 65.7% of lung tumors, respectively; p53 had a positive correlation with regional lymph node metastasis and advanced tumor stage, while an inverse correlation between the expression of pRB and p53 was found. By multivariate analysis, p53 expression and pathological stage were independent prognostic factors, while pRB status did not represent a prognostic marker (Lee et al., 1999b).

D. CDKN2A ($p16^{INK4a}$)

Members of the INK4 protein family inhibit cdk4 and cdk6-mediated phosphorylation of pRB; inactivation of $p16^{INK4a}$ in tumors expressing wild-type pRB is required for malignant cells to enter the *S* phase or escape senescence (Shapiro et al., 1998). The occurrence of $p16^{INK4a}$ lesions is second only to p53 abnormalities in human cancer and is a frequent event in premalignant lesions of the upper digestive tract (Wong et al., 2001). The *CDKN2A* gene is inactivated by a two-hit mechanism that can involve CpG island methylation, 9p21 LOH, mutation, or homozygous deletion (Pines, 1997; Sherbet and Lakshmi, 1997). *CDKN2A* is a tumor-suppressor gene that regulates cell-cycle progression through a G_1/S restriction point by inhibiting CDK4 and CDK6/cyclin D-mediated phosphorylation of pRB. The *CDKN2A* locus on chromosome 9p21 encodes two proteins translated by alternative splicing of mRNA; the α-transcript, $p16^{INK4a}$, which inhibits phosphorylation of pRB through cyclin D1/CDK4; and the β-transcript, $p14^{ARF}$, the binding of which to MDM2 stabilizes it and increases the availability of wild-type p53 (Pines, 1997; Sherbet and Lakshmi, 1997). Homozygous deletion of $p16^{INK4a}$ and the less frequent promoter hypermethylation disrupt the pathway of pRB/cyclin D1/CDK4, whereas homozygous deletion and hypermethylation of $p14^{ARF}$ permit degradation of p53 exported by MDM2 no longer sequestered in the nucleus. In addition to this, amplification of MDM2 is an alternative way of inactivating p53 (Pines, 1997; Sherbet and Lakshmi, 1997). The analysis of $p16^{INK4a}$, $p14^{ARF}$, and p53 in 38 primary NSCLC specimens (19 adenocarcinomas and 19 squamous carcinomas) showed that $p16^{INK4a}$ was inactivated in 58% of tumors by homozygous deletions, promoter hypermethylation, and point mutation in exon 2 (Sanchez-Cespedes et al., 1999). Fourteen tumors had simultaneous $p16^{INK4a}$ and $p14^{ARF}$ inactivation, most frequently because of homozygous deletions extending into the INK4a/ARF locus (Sanchez-Cespedes et al., 1999). Additional studies demonstrated abnormal $p16^{INK4a}$ expres-

sion in 46% of NSCLCs examined. No relationship was observed between $p16^{INK4a}$ abnormal staining and various clinico-pathological parameters (Spanakis et al., 1999). Deletions of *CDKN2A* may represent a predominant mechanism of gene inactivation. LOH was also observed at the D9S162 (35%) and D9S126 (38%) loci, which lie 6 cM and 4 cM, respectively, from the area that encodes $p16^{INK4a}$, implying that other tumor-suppressor genes may reside in this region (Spanakis et al., 1999). A study provided evidence that aberrant expression of $p16^{INK4a}$ and pRB was observed in 33 (49%) and 27 (40%) of 68 NSCLCs, respectively. Molecular analysis revealed that deletions and transcriptional silencing by methylation were the main mechanisms of *CDKN2*/$p16^{INK4a}$ inactivation in NSCLCs (Gorgoulis et al., 1998). Multiple genetic abnormalities are frequently observed in NSCLCs, including overexpression of p53 and MDM2, abnormal pRB expression, and elevated levels of MDM2 and p53 (Gorgoulis et al., 1998). Moreover, deregulated expression of $p16^{INK4a}$, pRB, p53, and MDM2 occurred in a large proportion (43%) of NSCLCs. This finding was not related to the clinical stage of the tumors, suggesting that abnormalities of this network occur early in the development of a subset of NSCLCs (Gorgoulis et al., 1998). Finally, smoking was associated with LOH and microsatellite instability at the 9p21–22 locus as well as with aberrant expression of $p16^{INK4a}$/pRB and overexpression of p53/MDM2 (Gorgoulis et al., 1998).

E. MYC

Overexpression of dominant oncogenes plays a role in tumor progression and it appears to be a late event in lung cancer pathogenesis. The *MYC* family of oncogenes produces proteins that, when expressed in the nucleus, lead to cell proliferation. *MYC* overexpression occurs in the vast majority of SCLCs but is rare in NSCLCs (Salgia and Skarin, 1998; Bunn et al., 2000). The *c-MYC* gene encodes a transcription factor that heterodimerizes with a partner protein, termed Max, to regulate gene expression by binding to specific DNA sequences. Max also heterodimerizes with the Mad family of proteins to repress transcription, antagonize c-MYC, and promote cellular differentiation (Grandori et al., 2000; Amati et al., 2001). These DNA-bound heterodimers recruit coactivator or corepressor complexes that generate alterations in chromatin structure, which in turn modulate transcription. Initial identification of target genes suggests that the network regulates loci involved in the cell cycle, growth, life span, and cell morphology. Because c-MYC and Mad proteins are expressed in response to diverse signaling pathways, the network can be viewed as a functional module that acts to convert environmental signals into specific gene-regulatory programs (Grandori et al., 2000; Amati et al., 2001). The most frequent genetic alteration that affects *c-MYC* is gene amplification, which is found in 8 to 20% of NSCLCs (Salgia and Skarin, 1998; Wistuba et al., 2001).

F. Bcl-2

The *Bcl-2* gene was identified as an oncogene in follicular lymphoma associated with the chromosomal translocation between chromosomes 18 and 14. The *Bcl-2* gene is located on chromosome 18q21 and the Bcl-2 protein is associated with the inner and outer mitochondrial membranes, as well as with the nuclear membrane and endoplasmic reticulum. Bcl-2 extends cell survival and inhibits drug-induced apoptosis (for review, see Loni et al., 2001) by multiple mechanisms, including inhibition of p53-dependent apoptosis triggered by Bax; however, its protective effect may be lost if the protein is phosphorylated. In some tumor cells expressing high levels of wild-type p53, the pro-apoptotic protein Bax is up-regulated, while Bcl-2 is down-regulated (Miyashita et al., 1994).

Abnormal expression of the *Bcl-2* gene product has been found in a wide variety of tumors, including NSCLC. The analysis of 116 tumor specimens from surgically resected NSCLC revealed that 34% of them showed Bcl-2 expression, which was found more frequently in males than females, and in smokers (Dosaka-Akita et al., 1999). Bcl-2 expression is observed more frequently in squamous cell carcinomas (53%) than in adenocarcinomas (22%) and, among squamous cell carcinomas, in pathological stage I tumors (85%) than in stage II and III cancers (42%). Bcl-2 expression did not correlate with p53 protein status and tumors metastatic to regional lymph nodes were most frequently Bcl-2-negative (Dosaka-Akita et al., 1999). Bcl-2 status appears not to influence the 5-year survival, since patients with Bcl-2-positive or -negative tumors had a similar survival rate; these data indicate that Bcl-2 expression is frequently observed in squamous cell carcinomas at an early stage, and that it does not predict the prognosis of patients with NSCLC (Dosaka-Akita et al., 1999). The immunohistochemical examination of apoptosis-regulating proteins, including Bcl-2, Mcl-1, Bax, Bak, and p53, revealed that tumor specimens positive for the anti-apoptotic proteins Bcl-2 and Mcl-1 were 31% and 58% of the cases evaluated, respectively, whereas the pro-apoptotic proteins Bax and Bak were found in 47% and 58% of the samples (Borner et al., 1999). The immunopositivity of p53 was detected in 61% of the samples; the expression of Bcl-2 and p53, and that of Mcl-1 and Bax, showed a significant positive association, whereas the expression of Bax was inversely related to p53. The expression of Bcl-2 had a negative influence on relapse-free survival in this population of resected NSCLC patients, but only subjects with p53-positive tumors developed metastases during follow-up (Borner et al., 1999).

The combination of Bcl-2-positivity/p53-negativity in NSCLCs was associated with the worst survival rate (Dingemans et al., 1999), and a comparison of long-term

versus short-term survivors after surgical resection of stage III squamous cell carcinomas demonstrated that factors involved in apoptosis, including p53 and Bcl-2, were up-regulated in subjects belonging to the long survival group (Mattern et al., 2002). In another study of 238 cases of NSCLC (203 squamous cell carcinomas and 35 adenocarcinomas), $p185^{HER\text{-}2/neu}$ and Bcl-2 were expressed at high levels in 42 and 71.8% of NSCLCs, respectively. Univariate analysis demonstrated that Bcl-2 expression was significantly associated with a poor prognosis, as it was the coexpression of Bcl-2 with $p185^{HER\text{-}2/neu}$, p53, and p21ras. However, only the combination of Bcl-2/$p185^{HER\text{-}2/neu}$ expression was an independent marker of poor prognosis on multivariate analysis (hazard ratio = 1.91) (Kim et al., 1998b). A recent study of 102 patients with NSCLC demonstrated Bcl-2 protein overexpression in 48% of tissue specimens, with no statistical association with p53 overexpression or mutation (Laudanski et al., 2001). This study also confirmed that, in a multivariate analysis, only *TP53* gene mutations seem to have a strong and independent effect on prognosis (Laudanski et al., 2001). Bronchial biopsies from 60 lung cancer patients were found p53- (43.3%), $p21^{Waf1/Cip1}$- (60%), and Bcl-2- (33.3%) positive. While single-protein expression was not associated with prognosis, the combined immunophenotype p53(−)/$p21^{Waf1/Cip1}$ (++)/Bcl-2 (−) predicted longer survival ($P = 0.03$). The authors concluded that p53 and Bcl-2 alterations may happen early in bronchial carcinogenesis and that the absence of these alterations in combination with $p21^{Waf1/Cip1}$ overexpression may be associated with a less aggressive tumor behavior (Kalomenidis et al., 2001). In surgical samples of NSCLC, detectable Bcl-2 was shown by immunohistochemistry in 20 of 107 (19%) cases and this finding was associated with squamous cell histology. An inverse relationship was found between Bcl-2/vascular grade and Bcl-2/$p185^{HER\text{-}2/neu}$, while no relationships were found between p53 and EGFR expression and Bcl-2, $p185^{HER\text{-}2/neu}$, or vascular grade. The improved prognosis of Bcl-2-positive NSCLC may be related to low tumor vascularization; since normal lung epithelium expresses Bcl-2, tumor progression may involve the loss of Bcl-2 followed by activation of HER-2/neu or increase in tumor vascularization (Koukourakis et al., 1997). The immunohistochemical analysis of 216 NSCLC specimens from T1–2 and N0–1 patients confirmed the inverse relationship between Bcl-2 and HER-2/neu and demonstrated that high thymidine phosphorylase and vascular endothelial growth factor (VEGF) reactivity was statistically related to loss of Bcl-2 expression ($P < 0.01$). Thus, this study provided additional evidence that the *Bcl-2* gene has a suppressive function on genes involved in angiogenesis (VEGF and thymidine phosphorylase) and cell proliferation (HER-2/neu) in NSCLC (Koukourakis et al., 1999). Additional data in line with these findings were provided in a later study (Boldrini et al., 2000).

G. FHIT

FHIT is a tumor-suppressor gene located at 3p14.2, a region frequently lost in multiple tumor types, and abnormalities of *FHIT* have been found frequently in NSCLC. To investigate whether *FHIT* inactivation plays a role in early lung tumorigenesis, pFHIT immunohistochemistry was performed in tumors from 87 patients with stage I NSCLC and in 372 bronchial biopsy specimens from 86 chronic smokers without evidence of malignancy (Tseng et al., 1999). It was found that 49% of NSCLC specimens demonstrated significantly decreased or lack of staining for pFHIT. However, pFHIT expression status was not significantly associated with disease-free or overall survivals (Tseng et al., 1999). The analysis of a subset of 76 specimens on which microsatellite analysis at the *FHIT* locus was performed did not show a strong association between LOH at *FHIT* and protein levels, suggesting the presence of complex mechanisms of *FHIT* inactivation (Tseng et al., 1999). Of 372 bronchial biopsies from chronic smokers, 86 biopsies (23%) exhibited decreased or lack of pFHIT expression. Loss of pFHIT was significantly higher in bronchial metaplastic lesions (47%) than in histologically normal bronchial epithelium (20%; $P < 0.001$) (Tseng et al., 1999). Smokers with a metaplasia index of >15% had a higher frequency of loss of pFHIT expression than those with a metaplasia index of ≤15% ($P = 0.015$). These data indicate that pFHIT expression is significantly reduced in a substantial number of early-stage NSCLC and preneoplastic lesions in chronic smokers. The association between cigarette smoking and pFHIT expression suggests a role for *FHIT* in the initiation of smoking-related lung tumorigenesis (Tseng et al., 1999).

H. Epidermal Growth Factor Receptors

Epidermal growth factor (EGF) was one of the first growth factors to be discovered and is the prototype of a large family of closely related peptides that includes TGF-α, amphiregulin, heparin-binding EGF, and β-cellulin (Heldin and Rönnstrand, 1997; Sherbet and Lakshmi, 1997; Ciardiello and Tortora, 2001). Among these growth factors, TGF-α has been identified as a key modulator in the process of cell proliferation in both normal and malignant epithelial cells. TGF-α binds to its specific cell membrane receptor, the EGFR, leading to the activation of the EGFR tyrosine kinase enzymatic activity that triggers the intracellular signaling pathway. The EGFR is part of a subfamily of four closely related proteins: EGFR (also called ErbB-1), HER-2/neu (ErbB-2), HER-3 (ErbB-3), and HER-4 (ErbB-4) (Heldin and Rönnstrand, 1997; Sherbet and Lakshmi, 1997; Ciardiello and Tortora, 2001). The *HER-2/neu* gene is located on chromosome 17p21; it codes for a 185-kDa transmembrane glycoprotein ($p185^{HER\text{-}2/neu}$) related to EGFR. *HER-2/neu* is activated by a point mutation that results in the change of amino acid residue 664 from

valine to glutamic acid, and this change is associated with its ability to transform cells. Alterations and amplifications of this gene have been reported in a variety of human cancers such as NSCLC (Heldin and Rönnstrand, 1997; Sherbet and Lakshmi, 1997; Ciardiello and Tortora, 2001). The receptors exist as inactive monomers, which dimerize after ligand activation. This causes homodimerization or heterodimerization between EGFR and other members of the Erb receptor family. After ligand-binding the tyrosine kinase intracellular domain of the receptor is activated and undergoes autophosphorylation, which initiates a cascade of intracellular events. The signaling pathway involves activation of p21ras and MAPK, which activate, in turn, several nuclear proteins, including cyclin D1. EGFR signaling is not only critical for cell proliferation; indeed, several studies have demonstrated that EGFR-mediated signals also contribute to other processes that are crucial to cancer progression, including angiogenesis, metastasis, and inhibition of apoptosis (Heldin and Rönnstrand, 1997; Sherbet and Lakshmi, 1997; Ciardiello and Tortora, 2001). These effects may be explained, at least in part, by the evidence that clinical specimens of NSCLCs overexpressing both EGFR and $p185^{HER\text{-}2/neu}$ also have higher levels of the inducible isoform of cyclooxygenase (Cox-2) than those without concomitant overexpression of these proteins (Niki et al., 2002). Furthermore, Cox-2 mRNA levels correlate with both $p185^{HER\text{-}2/neu}$ expression and a phosphorylated form of MAPK/ERK in lung cancer cell lines, and the addition of TGF-α increased Cox-2 mRNA levels in cells. These results suggest that EGFR signaling is involved in the enhanced expression of Cox-2 in lung adenocarcinomas (Niki et al., 2002). The 11p15 mucin genes (MUC2, MUC5AC, MUC5B, and MUC6) possess a cell-specific pattern of expression in normal lung that is altered during neoplastic transformation. Studies on the mucoepidermoid NCI-H292 lung cancer cell line demonstrated that treatment with EGF and TGF-α resulted in a strong increase of MUC2 and MUC5AC mRNAs levels, promoter activity, and apomucin expression. Up-regulation of MUC2 and MUC5AC genes was associated with activation of the EGFR/Ras/Raf/MEK signaling pathway leading to cell proliferation (Perrais et al., 2002). Abnormal activation of autocrine pathways mediated by EGFR in cancer cells may be attributed to several mechanisms, such as overexpression of EGFR, increased availability of ligands, decreased receptor turnover, decreased phosphatase activity, and aberrant receptors, including *EGFR* gene alterations. In this context, the most common *EGFR* mutant found in human cancer is *EGFRvIII*. The rearranged *EGFRvIII* gene is often amplified, thus resulting in tumor cells overexpressing EGFRvIII protein, a truncated EGFR that lacks domains I and II of the extracellular domain, and is not capable of ligand binding (Heldin and Rönnstrand, 1997; Sherbet and Lakshmi, 1997; Ciardiello and Tortora, 2001). However, it has a constitutively activated tyrosine kinase domain that stimulates cell proliferation independently of ligand interaction.

EGFR is overexpressed in NSCLC, in which it is generally associated with advanced disease and poor prognosis. EGFR overexpression has been associated with resistance to cytotoxic agents, including cisplatin (Chen et al., 2000), and the ErbB receptor tyrosine kinase network stimulates cytoprotective p70 S6 kinase and Akt activity in response to clinically relevant doses of ionizing radiation (Contessa et al., 2002). The combined *HER-2/neu* and *EGFR* overexpression in the same aneuploid cells defines a genetic evolutionary sequence that is common to NSCLC; later steps in this sequence include *RAS* and *c-MYC* overexpression (Shackney and Shankey, 1997). The immunohistochemical analysis of 408 stage I NSCLC specimens for p53, Bcl-2, $p185^{HER\text{-}2/neu}$, KI-67, pRB, and EGFR demonstrated that among men, the molecular marker associated with decreased cancer-specific survival is $p185^{HER\text{-}2/neu}$, while among women, these are p53 and pRB (D'Amico et al., 2000). The study of the association between *EGFR* and *HER-2/neu* gene expression and survival in primary tumor and matching nonmalignant tissues from 83 patients with NSCLC demonstrated that *EGFR* and *HER-2/neu* mRNA were detectable in all specimens analyzed (Brabender et al., 2001). High *HER-2/neu* and *EGFR* expression was detected in 29 (34.9%) and 28 (33.7%) patients, respectively, while high *HER-2/neu* and *EGFR* coexpression was detectable in 14 (16.9%) subjects. *HER-2/neu* up-regulation was associated with shorter survival ($P = 0.004$), whereas high *EGFR* expression showed a trend toward reduced survival (Brabender et al., 2001). Multivariate analysis demonstrated that high expression of *HER-2/neu* and combined *EGFR-HER-2/neu* were significant and independent unfavorable prognostic factors, thus indicating that *HER-2/neu* and *EGFR* play a crucial role in the biological behavior of NSCLCs (Brabender et al., 2001). A recent study provided additional evidence that coexpression of EGFR and matrix metalloproteinase-9 (MMP-9), microvessel density, and Bcl-2 were independent prognostic variables that allowed prediction of patient outcome independent of surgical stage (O'Byrne et al., 2001).

I. Multidrug Resistance Proteins

NSCLC is considered to be a chemotherapy-refractory malignancy because of the lack of clinical efficacy of single-agent therapy. Chemoresistance thus remains the major obstacle to successful therapy of NSCLC, and one of the reasons for such a biological profile is the expression in tumor tissue of three protein families involved in multidrug resistance: 1) P-glycoprotein (P-gp), a 170-kDa protein encoded by the *MDR1* gene; 2) the multidrug resistance-associated protein (MRP) family, a group of at least six members, the best characterized of which is a 190-kDa protein (ABCC1, MRP); and 3) the

vault-transporter lung resistance protein (LRP), a 110-kDa protein encoded by the *LRP* gene (Borst et al., 1999; Scheffer et al., 2000; Tan et al., 2000; Young et al., 2001). Both P-gp and MRP are membrane-associated transport proteins belonging to the large and ancient ATP-binding cassette (ABC) superfamily; P-gp contains two multispanning transmembrane domains compared with the three that are present in MRP, which requires reduced glutathione for its function (Scheffer et al., 2000; Tan et al., 2000). LRP is located intracellularly and appears to be involved in the resistance to cisplatin (Berger et al., 2000). P-gp and MRP are most involved in the transport of taxanes, anthracyclines, vinca alkaloids, and epipodophyllotoxins to the outside of the membrane (Tan et al., 2000), while MRP is also involved in cisplatin resistance (Young et al., 2001).

The expression of drug-resistant proteins is expected to be highly variable, depending on treatment status and the type of drug administered in combination regimens. Unfortunately, there are few studies published in the literature addressing this point. In 15 cell lines unselected with respect to chemotherapeutic agents, MRP was constitutively expressed, with markedly varying intensity (Berger et al., 1997). Two cell lines expressed high MRP protein levels without amplification of the *MRP* gene and a significant correlation between MRP expression and chemoresistance toward doxorubicin, etoposide, and vinblastine was observed (Berger et al., 1997). The immunohistochemical analysis of paraffin-embedded tissue from 27 cases of untreated NSCLC showed the presence of P-gp in >5% of cells in only 3 of 27 cases, and MRP in 5 of 27 tumors (Kreisholt et al., 1998). A study of surgical tissue samples from 84 untreated NSCLCs analyzed the levels of *MDR1* mRNA and demonstrated that 15% of tumors were positive for the *MDR1* gene, but the level was low in all samples except for one adenocarcinoma, which expressed high levels of MDR1. Gene expression in these tumors was unrelated to pathologic factors such as histologic type, pathologic stage, and tumor size (Oka et al., 1997). Among 36 samples of surgically resected NSCLC squamous cell and adenocarcinomas had higher LRP expression than large cell undifferentiated and mixed tumors, while MRP expression was detected in few specimens (Dingemans et al., 1996). Contrasting results were obtained in studies aimed at establishing a relationship between drug sensitivity and expression of proteins involved in drug resistance. Using a large panel of unselected cell lines, *MRP* mRNA expression was a poor predictor of drug sensitivity, at variance with *MDR1*, suggesting that other factors, including conjugating enzymes, glutathione levels, or other transporters, confound the MRP effect (Alvarez et al., 1998). In another study conducted on unselected NSCLC cell lines, the mRNA levels of *MRP* correlated with resistance to vincristine, etoposide, and cisplatin, thus implying that MRP may contribute to the drug resistance phenotype of lung cancer cells (Young et al., 1999). Detectable levels of MRP in most of the tumor mass was found in 87% of samples of untreated NSCLC. In a substantial proportion of adenocarcinomas (55%) and squamous cell carcinomas (28%), immunoreactivity approached that obtained with the highly multidrug-resistant cell line H69AR, from which the MRP was originally cloned (Wright et al., 1998). No potentially confounding correlation independent of its possible role in drug resistance was observed between MRP expression in untreated NSCLC and any clinicopathological parameter examined, including overall survival (Wright et al., 1998). The predictive value of MRP and LRP expression in NSCLC tumor biopsies with respect to treatment response was evaluated in 38 patients who had been subsequently treated with cisplatin/paclitaxel, cisplatin/teniposide or etoposide, or carboplatin/etoposide/ifosfamide. The study demonstrated that none of the investigated markers was related to overall response rate (Dingemans et al., 2001). In a study on NSCLC the frequency of expression of LRP, MRP, and P-gp in tumor tissue was 74.2%, 80.3%, and 37.9%, respectively, and was unrelated to cell differentiation and tumor staging. At variance with previous studies, there was lower expression of both LRP and MRP in chemo-responsive adenocarcinomas compared with unresponsive tumors; in squamous cell carcinomas, however, this was applicable to LRP expression only. Finally, coexpression of drug resistance-related genes adversely affected median survival of NSCLC patients (Wang et al., 2000).

V. Potential Role of Pharmacogenetics in Rational Therapeutic Decision

Pharmacogenetics is the study of how the responses of patients to drugs are affected by their genetic profile (Evans and Relling, 1999; Roses, 2000). The ability to select patients on the basis of the likelihood of response to a specific chemotherapeutic agent would avoid the empiricism dependent on the inability to match the most appropriate drug with the specific genetic profile of the tumor. Our increasing knowledge of the mechanisms of drug action, the identification of new drug targets, and the understanding of genetic factors that determine the response of individual patients may allow the design of drug treatments that are specifically targeted toward particular populations or that avoid genetic variability in therapeutic response.

The extent of genetic polymorphism in the human population indicates that pharmacogenetic variability is likely to be an important issue for most drugs (Danesi et al., 2001). The current application of pharmacogenetics to the treatment of cancer patients is still at the investigational stage and is mostly concerned with the screening of individuals for genetically determined defects in drug metabolism that are associated with severe, potentially life-threatening adverse drug reactions (Danesi et

al., 2001). Indeed, genetic polymorphism has been recognized as a major cause of unexpected toxicity after the administration of analogs of pyrimidines (5-fluorouracil), purines (6-mercaptopurine), and folic acids (methotrexate) as well as a number of natural products, including inhibitors of topoisomerase I and II (anthracyclines and camptothecins) (Danesi et al., 2001). Genes involved in biotransformation and detoxification of the abovementioned anticancer agents include dihydropyrimidine dehydrogenase, thiopurine methyltransferase, and UDP-glucuronosyl-transferase, while other enzymes are the targets of drug action (e.g., methylene-tetrahydrofolate reductase). The following chapters report on the available data from the scientific literature concerning the influence of genes that play a role in NSCLC tumor progression, including *RAS*, *TP53*, *RB*, *p16^{INK4a}*, *MYC*, *FHIT*, *Bcl-2*, *EGFR*, and multidrug-resistant superfamilies; on the chemotherapeutic activity of drugs currently used for the medical management of NSCLC, including platinum compounds (cisplatin and carboplatin), taxanes (paclitaxel and docetaxel), gemcitabine, epipodophyllotoxins (etoposide), vinca alkaloids (vinorelbine), alkylating agents (ifosfamide and cyclophosphamide); and new agents, including camptothecin analogs (topotecan and irinotecan), inhibitors of EGFR, and novel folic acid analogs. An overview of the best-characterized interactions between genes and anticancer drugs in NSCLC is reported in Fig. 1.

VI. Influence of Genetic Profile of Non-Small Cell Lung Cancer on Drug Activity

A. Platinum Compounds

Cisplatin and carboplatin (Fig. 2) are alkylating agents widely used in cancer chemotherapy. Response to cisplatin is mainly mediated through a p53-dependent apoptotic pathway. Most studies have reported that lung cancer cells having wild-type p53 are more sensitive to cisplatin than cells having either mutant or null p53. The overexpression of p53 induced by cisplatin may trigger apoptotic pathways via transactivation of the *Bax* gene (Yoon et al., 2001). A study examined the introduction of a mutant p53 gene into the NCI-H460 cell line, which carries a wild-type p53 gene, and the wild-type p53 gene into NCI-H1437, NCI-H727, NCI-H441, and NCI-H1299 cells which, in turn, bear a mutant p53 gene (Lai et al., 2000). The NCI-H1437 cell line transfected with a wild-type p53 gene showed a dramatic increase in susceptibility to cisplatin compared to untransfected NCI-H1437 cells. An increase in chemosensitivity was also observed in wild-type p53 transfectants of NCI-H727, NCI-H441, and NCI-H1299 cells (Lai et al., 2000). In contrast, loss of chemosensitivity and lack of p53-mediated DNA apoptotic degradation in response to anticancer agents were observed in NCI-H460 cells transfected with mutant p53. These observations suggest that p53 gene status modulates the extent of chemosensitivity and the occurrence of apoptosis by cisplatin (Lai et al., 2000). The infection of recombinant adenovirus expressing wild-type p53 to lung cancer cells that harbor a mutant p53 gene improves their response to cisplatin (Horio et al., 2000). Experimental studies on the effects of wild-type p53 gene transfer in combination with various anticancer agents on the human pulmonary squamous cell carcinoma cell line NCI-H157 and the human pulmonary large cell carcinoma cell line NCI-H1299 provided evidence that cisplatin showed a high degree of effectiveness and an additive effect with p53 transduction on NCI-H157 and NCI-H1299 cells (Osaki et al.,

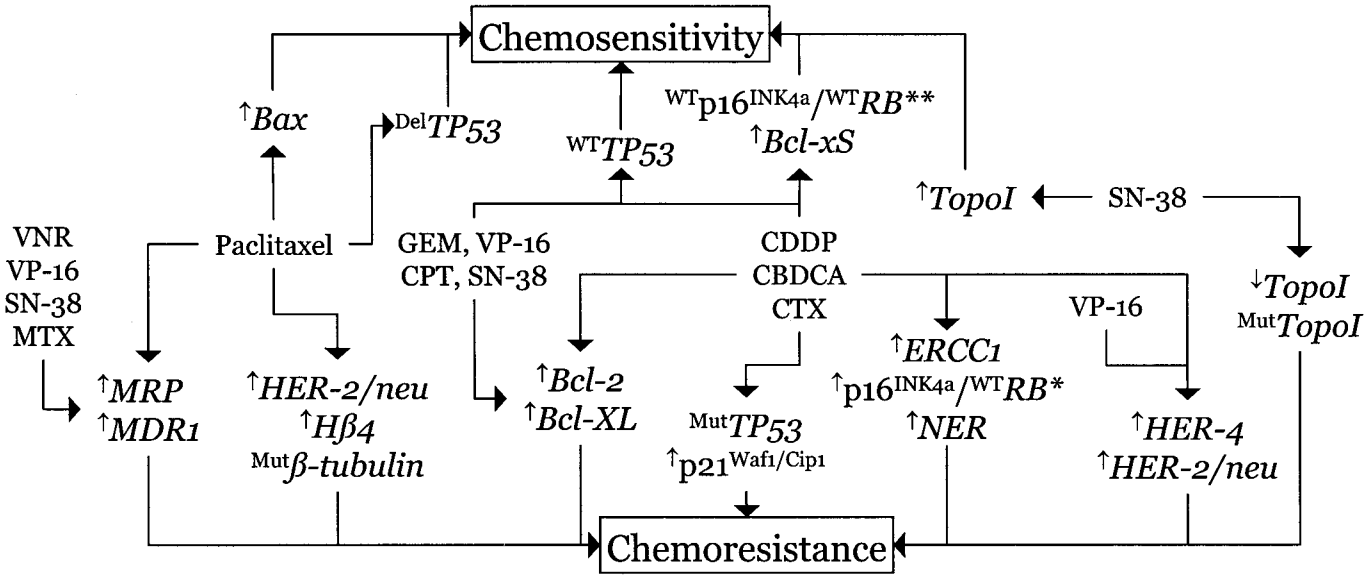

FIG 1. Schematic representation of interactions between genes involved in lung tumorigenesis and NSCLC progression and anticancer agents commonly used for the medical management of NSCLC. ↑, overexpression; ↓, downregulation; Mut, mutation; Del, deletion; WT, wild-type; VNR, vinorelbine; VP-16, etoposide; SN-38, 7-ethyl-10-hydroxycamptothecin; MTX, methotrexate; GEM, gemcitabine; CPT, camptothecin; CDDP, cisplatin; CBDCA, carboplatin; CTX, cyclophosphamide; *, data from in vitro studies; **, data from in vivo studies.

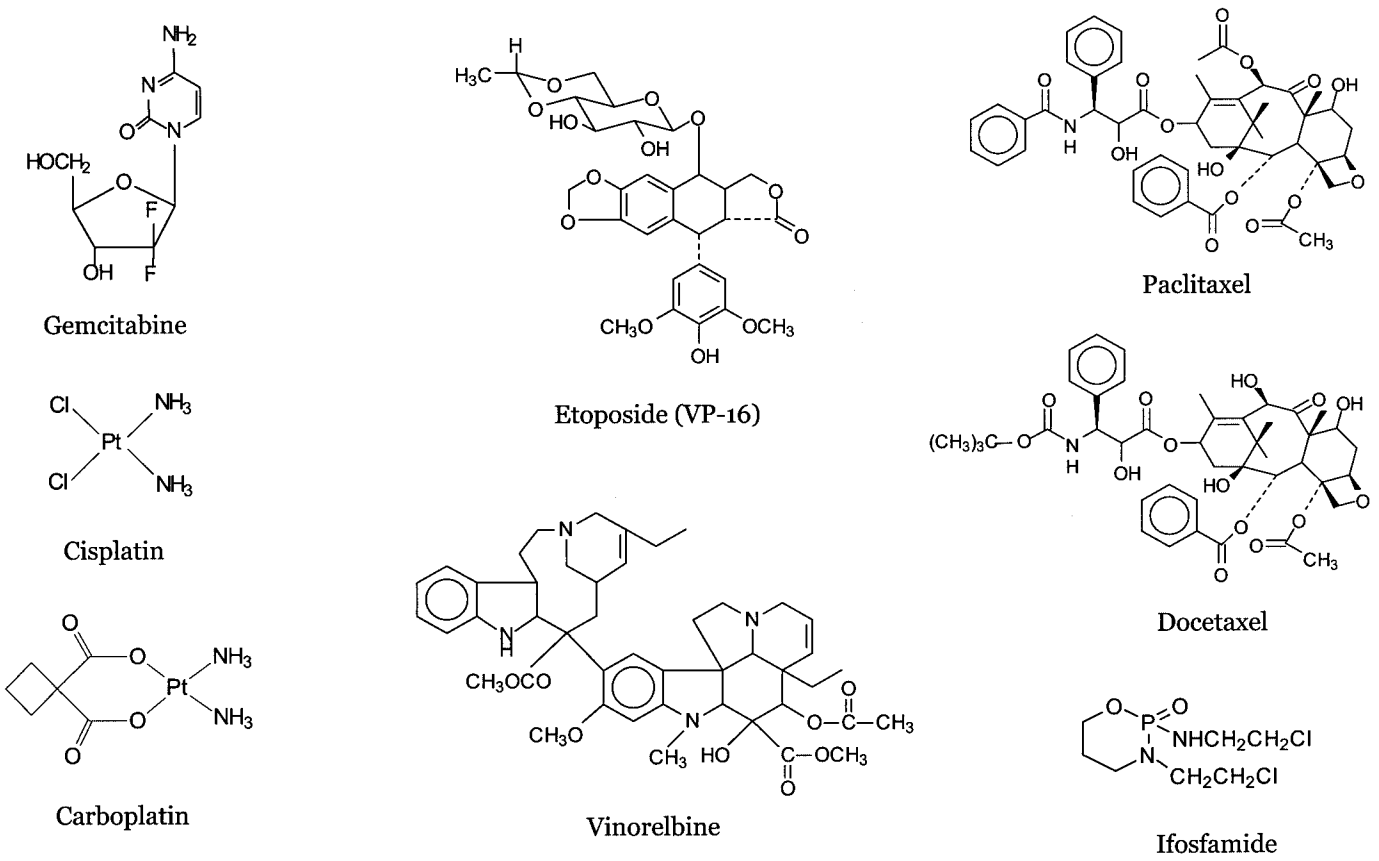

FIG 2. Chemical structures of currently used agents for the treatment of NSCLC.

2000). These results indicate that cisplatin would be a candidate drug for the combination of chemotherapy and gene therapy for NSCLC (Osaki et al., 2000). Since it appears well established that p53 and DNA-damaging agents such as cisplatin work synergistically to induce apoptosis in cancer cells (Roth et al., 1999), patients with NSCLC in complete or partial remission were compared with those with stable or progressive disease with respect to *TP53* genotype and overall survival. Mutations in the *TP53* gene were detected by sequencing of exons 2–11 (Kandioler-Eckersberger et al., 1999). A normal *TP53* genotype proved to be significantly associated with major response to chemotherapy ($P < 0.001$). However, no association was found between p53 protein expression and *TP53* genotype (Kandioler-Eckersberger et al., 1999). A normal *TP53* genotype was found to be highly sensitive in predicting response to treatment, whereas a mutant *TP53* predicted lack of response. The difference in overall survival was significant comparing patients with a normal *TP53*, and responding to chemotherapy, with patients showing mutant *TP53* with a disease resistant to chemotherapy (Kandioler-Eckersberger et al., 1999). Therefore, a direct link between wild-type *TP53* and response to cisplatin-based induction treatment and between mutant genotype and resistance to treatment was found, whereas the p53 immunohistochemical result was predictive of neither (Kandioler-Eckersberger et al., 1999). These data are partially in contrast with another study, in which immunohistochemical analysis demonstrated a 70% concordance between overexpression of p53 protein and mutation in *TP53* (Brattstrom et al., 1998). Indeed, tumor specimens from 52 patients with stage IIIA NSCLC who were enrolled in a prospective clinical trial of cisplatin-based induction chemotherapy followed by surgical resection were examined for p53 expression which, in turn, was correlated with clinical and pathological response (Rusch et al., 1995). No relationship was established between p53 expression and activity of the treatment because 47 of 52 patients had a major response (Rusch et al., 1995). However, a significant association was observed between aberrant p53 expression and resistance to chemotherapy as assessed by pathological response. Only 3 of 20 patients whose tumors exhibited a high level of p53 staining experienced a major pathological response to chemotherapy, while just 7 of 52 cases examined before and after cisplatin-based chemotherapy exhibited a change in the level of p53 expression (Rusch et al., 1995). These results indicate that cisplatin alters p53 expression infrequently and suggest a direct link between aberrant p53 expression and resistance to cisplatin-based chemotherapy in NSCLC (Rusch et al., 1995). Additional clinical studies provided evidence that wild-type p53 expression in tumors correlates with both

good response to cisplatin-based chemotherapy and better survival of patients with advanced NSCLC (Oshita et al., 2000). Resected tumors from 18 patients with recurrent NSCLC who had undergone complete resection and received chemotherapy after the initial tumor recurrence were subjected to p53 immunostaining. Histological examination of the resected tumors revealed 11 adenocarcinomas, 6 squamous cell carcinomas, and 1 adenosquamous cell carcinoma. Group 1 displayed $\geq$50% (n = 9) and group 2 >50% (n = 9) p53-immunopositive tumor tissues, and all patients received cisplatin-based chemotherapy after recurrence (Oshita et al., 2000). None of the patients in group 1 achieved response to chemotherapy, whereas five subjects in group 2 achieved a complete and/or partial response (56%), respectively (Oshita et al., 2000). The time to recurrence after tumor resection of group 2 was significantly better than that of group 1 (log-rank P = 0.019; Wilcoxon P = 0.042), and survival of group 2 after chemotherapy was also significantly longer than that of group 1 (log-rank P = 0.023; Wilcoxon P = 0.034) (Oshita et al., 2000). It is suggested that high p53 expression levels in tumors correlate with both good response to cisplatin-based chemotherapy and better survival of patients with advanced NSCLC (Oshita et al., 2000). The gene encoding glycosyl-phosphatidyl-inositol-anchored molecule-like protein (*GML*) is induced by wild-type p53 and the analysis of 30 surgically resected NSCLC specimens revealed that *GML* expression was detectable in nine samples (30%), and its incidence was significantly higher in p53-negative or wild-type p53 tissues examined by immunohistochemistry (Higashiyama et al., 2000). In particular, among p53-negative tumors, those with *GML* gene expression showed a significantly better sensitivity to cisplatin; furthermore, a good response to cisplatin-based chemotherapy in NSCLC patients with tumor residue or recurrence was observed only in those with p53-negative tissue with detectable *GML* gene expression (Higashiyama et al., 2000). It appears that in p53-negative NSCLCs *GML* is generally expressed and it is associated with good sensitivity to cisplatin, thus representing a predictor of response (Higashiyama et al., 2000). On the basis of the role of p53 in the chemosensitivity to anticancer agents, recombinant adenovirus-mediated transfer of the wild-type p53 gene was devised as a potentially useful strategy for gene therapy; indeed, gene transfer into monolayer cultures or multicellular tumor spheroids of the human NSCLC cell line NCI-H358, which has homozygous deletion of p53, markedly increased the cellular sensitivity of these cells to the chemotherapeutic effect of cisplatin (Fujiwara et al., 1994). Treated cells underwent apoptosis, and direct injection of the p53-adenovirus construct into H358 tumors implanted s.c. into athymic nu/nu mice, followed by i.p. administration of cisplatin, induced massive apoptotic death of the tumors (Fujiwara et al., 1994). These results provide support for the clinical application of regimens combining gene replacement with replication-deficient wild-type p53 adenovirus and DNA-damaging drugs for the treatment of NSCLC (Fujiwara et al., 1994). In a clinical study aimed at determining the safety and activity of adenovirus-mediated *TP53* gene transfer in tumor tissue followed by cisplatin administration in patients with advanced NSCLC and abnormal p53 function, subjects were administered intravenous cisplatin 80 mg/m^2 on day 1 and adenoviral vector carrying wild-type *TP53* on day 4 every 4 weeks, for a total of up to six courses (Nemunaitis et al., 2000). Transient fever related to p53 vector administration developed in 8 of 24 patients. The combination of gene transduction with chemotherapy proved to be clinically active, since 17 patients achieved stable disease, 2 patients had partial response, 4 patients had progressive disease, and 1 patient was not assessable (Nemunaitis et al., 2000). However, in a following study, the clinical application of intratumoral adenoviral *TP53* gene therapy failed to provide additional benefit in patients treated with an effective first-line chemotherapy for advanced NSCLC (Schuler et al., 2001). Indeed, *TP53* gene therapy was examined in 25 patients undergoing first-line chemotherapy for advanced NSCLC, including carboplatin at AUC6 plus paclitaxel 175 mg/m^2 (day 1) or cisplatin 100 mg/m^2 (day 1) plus vinorelbine 25 mg/m^2 (days 1, 8, 15, and 22), in combination with intratumoral injection on day 1 of 7.5×10^{12} particles of SCH 58500, a recombinant adenovirus carrying wild-type *TP53*. No difference was observed between the response rate of lesions treated with *TP53* gene therapy in addition to chemotherapy (52% objective responses) and tumors treated with chemotherapy alone (48% objective responses) (Schuler et al., 2001). Subgroup analysis according to the chemotherapy regimens revealed evidence for increased mean local tumor regressions in response to *TP53* gene therapy in patients receiving cisplatin plus vinorelbine, but not in patients receiving carboplatin plus paclitaxel (Schuler et al., 2001). There was no survival difference between the two chemotherapy regimens, and the median survival of the cohort was 10.5 months (1-year survival, 44%) (Schuler et al., 2001). Furthermore, in 27 NSCLC patients treated with concomitant daily low-dose cisplatin and radiotherapy, p53 expression showed no relationship with outcome (Van de Vaart et al., 2000).

The study of pRB expression in 171 cell lines derived from patients with lung malignancies, including NSCLC, revealed absent or aberrant pRB protein expression in 12 of 80 NSCLCs. A stable, hypophosphorylated mutant pRB was detected in 3 NSCLC samples (Shimizu et al., 1994). Analysis of the matched clinical data showed no associations between *RB* status and age, sex, extent of disease, performance status, smoking history, and previous treatment. In addition, retrospective analysis showed no consistent correlation of pRB with best clinical response, overall survival, or in vitro chemotherapeutic drug sensitivity (Shimizu et al., 1994). A

further investigation of the correlation of pRB expression levels and chemosensitivity of a panel of NSCLC cell lines bearing a wild-type *RB* provided evidence of a significant correlation between chemosensitivity and high levels of RB protein (Yamamoto et al., 1998).

The relationship between chemoresistance and the presence of *RAS* point mutations was investigated using a panel of 20 NSCLC cell lines established from untreated patients. The 50% inhibitory concentration (IC_{50}) values for cisplatin were not significantly different in the cell lines with or without mutated p21ras (Tsai et al., 1993). The same finding was observed in NCI-H82 human SCLC cells transduced with the *v-H-RAS* oncogene, which resulted in the conversion of the SCLC cellular phenotype into an NSCLC phenotype of the NCI-$H82^{Hras}$; p21ras expression was not associated with significant changes in cisplatin sensitivity (Kaufmann et al., 1995). Nonetheless, the farnesyltransferase inhibitor FTI-2153 was highly effective at suppressing post-translational activation of oncogenic p21ras as well as the constitutive activation of MAPK and human tumor cell proliferation in soft agar. In addition to this, the farnesyltransferase inhibitor FTI-2148 markedly inhibited the growth of the human lung adenocarcinoma A549 cells in nude mice in a dose-dependent manner, and the combination therapy of FTI-2148 with cisplatin resulted in a greater antitumor efficacy than monotherapy (Sun et al., 1999), possibly by interference with biologic activation of p21ras. Finally, the combination of the farnesyltransferase inhibitor SCH66336 with cisplatin produced antiproliferative effects that were additive or synergistic over a broad range of clinically achievable concentrations in A549 NSCLC cells (Adjei et al., 2001). Examination of the effect of various drug sequences in A549 cells revealed synergism when cells were exposed to SCH66336 and then cisplatin, and antagonism when drugs were administered in the opposite order. The additive and synergistic effects resulted in enhanced apoptosis with the SCH66336/cisplatin combination (Adjei et al., 2001). SCH66336 was ineffective in the formation or removal of cisplatin-DNA adducts, raising the possibility that SCH66336 affected survival of cisplatin-treated cells downstream of the DNA lesions (Adjei et al., 2001). Therefore, although *RAS* mutations have a modest effect on drug response of cancer cells, inhibition of p21ras activity significantly enhances the effect of cytotoxic agents administered in combination. In the clinical setting, to determine whether the course of the disease and the response to chemotherapy of patients with advanced adenocarcinoma of the lung is affected by *K-RAS* mutation, patients received chemotherapy with mesna, ifosfamide, carboplatin, and etoposide (Rodenhuis et al., 1997). The presence of *K-RAS* mutations could be established in 69 of 83 patients (83%). Patients with a *K-RAS* mutation in their tumor were more likely to have a close relative with lung cancer, but other clinical characteristics, such as pattern of metastases, response, and survival, were unrelated to the *K-RAS* genotype (Rodenhuis et al., 1997). Therefore, patients with advanced lung adenocarcinoma who harbor a *RAS* mutation may have major responses to chemotherapy and have similar progression-free and overall survival as patients with *RAS* mutation-negative tumors (Rodenhuis et al., 1997). Finally, the prognostic value of *K-RAS* mutations at codon 12 was evaluated in paraffin-embedded specimens of 40 patients with stage III NSCLC who underwent tumor resection after neoadjuvant treatment with two cycles of ifosfamide, carboplatin, and etoposide, and subsequent twice-daily radiotherapy with concurrent carboplatin and vindesine. A *K-RAS* codon 12-point mutation was found in 13 of 28 resection specimens (46%). Even after complete resection, the presence of a *K-RAS* mutation was a significant predictor for a poor progression-free survival (Broermann et al., 2002).

To study the interaction of genetic profile and mutational status of key tumor-suppressor genes with anticancer agents commonly used in NSCLC, the cytotoxicity induced by carboplatin alone and in combination with gemcitabine and paclitaxel was tested on the human cancer cell lines A549 ($p16^{INK4a}$ deleted, p53 wild-type, and pRB wild-type); Calu-1 ($p16^{INK4a}$ deleted, p53 deleted, and pRB wild-type); and NCI-H596 ($p16^{INK4a}$ wild-type, p53 mutated, and pRB deleted) (Edelman et al., 2001). The IC_{50} of carboplatin was similar on A549 and Calu-1 cell lines (27 and 36 μM), albeit it was much lower on the NCI-H596 cell line (2.9 μM). Although this study did not evaluate the statistical relevance of these findings and their possible link with the genetic pattern of cell lines, the findings suggest a possible influence of $p16^{INK4a}$ status on the chemosensitivity of cells that were less sensitive to carboplatin if $p16^{INK4a}$ was deleted. Carboplatin was synergistic with gemcitabine in all three cell lines, and the synergy was most pronounced in the A549 cells when gemcitabine preceded carboplatin (Edelman et al., 2001). Since the G_2 checkpoint has a key role in the response to DNA damage, a flow cytometry study investigated the expression of cyclin B1, cdc2, cdc25c, and DNA content in two lung cancer cell lines treated with cisplatin. In these cells, the G_2 arrest was associated with cdc2, cdc25c, cyclin B1, and $p16^{INK4a}$ increase; however, G_2 arrest after cisplatin treatment was associated with deregulation of cyclin B1, while other checkpoint proteins were not involved (Links et al., 1998). Calu-1 NSCLC cells, which bear a wild-type pRB and lack p53, do not arrest in G_1 phase following DNA damage; however, $p16^{INK4a}$ transduction restores the G_1 checkpoint arrest in response to treatment with cisplatin (Shapiro et al., 1998). This finding suggests that during tumor progression loss of $p16^{INK4A}$ expression may be necessary for cells with wild-type pRB to bypass the G_1 arrest and attain a fully transformed phenotype (Shapiro et al., 1998). In addition to this, the replacement of $p16^{INK4a}$ into the $p16^{INK4a}$-negative, pRB-positive bladder cancer cell line EJ

caused a profound inhibition of cell proliferation mediated by arrest in the G_1 phase of the cell cycle. In contrast, the $p16^{INK4a}$-positive, pRB-negative cell line J82 was unaffected by gene transduction (Grim et al., 1997). However, when adenovirally mediated $p16^{INK4a}$ replacement was followed by cisplatin treatment, a marked chemoresistance was observed in genetically modified cells (Grim et al., 1997).

The exposure of the human lung carcinoma cell line DLKP-SQ to clinically achievable concentrations of doxorubicin generated a resistant variant, DLKP-SQ/10p, which was found to be cross-resistant to P-gp- and MRP-transportable drugs, but slightly sensitized to carboplatin (NicAmhlaoibh et al., 1999). Analysis of mRNA levels in the resistant variant revealed overexpression of the anti-apoptotic Bcl-xL transcript and the pro-apoptotic Bax mRNA, but no alterations in Bcl-2 or Bag-1 mRNA levels (NicAmhlaoibh et al., 1999). Overexpression of the pro-apoptotic Bcl-xS gene in the DLKP-SQ/10p line may explain the increase in sensitivity to carboplatin, and indicates that the relative expression of different members of the Bcl-2 family of apoptosis-regulatory proteins may be important in determining sensitivity to drug-induced apoptosis (NicAmhlaoibh et al., 1999). After prolonged exposure of NCI-H460 NSCLC cells to increasing concentrations of cisplatin, a resistant subline was isolated (NCI-$H460^{CIS}$); these cells exhibited cross-resistance to other DNA damaging agents such as doxorubicin and etoposide (Yoon et al., 2001). Cisplatin exposure markedly increased p53 expression in parental cells but not in NCI-$H460^{CIS}$; without drug treatment, Bcl-2 and Bax were expressed in NCI-$H460^{CIS}$ cells, but not in parental cells (Yoon et al., 2001). These data suggest that p53 function is abrogated and Bax and Bcl-2 are up-regulated in NCI-$H460^{CIS}$ cells, thus providing an explanation of cisplatin resistance (Yoon et al., 2001). The protective role of Bcl-2 family members in cisplatin-induced cytotoxicity is further confirmed by the evidence that stable expression of Bcl-2 and Bcl-xL increases clonogenic survival of cells treated with cisplatin from 18% to 47–51%, suppresses cytochrome *c* release from mitochondria, caspase-8 activation, and occurrence of apoptosis by cisplatin treatment in the NCI-H460 NSCLC cell line (Ferreira et al., 2000a). Further confirmation of the importance of Bcl-2 in drug resistance against cisplatin has also been obtained in SCLC cell lines (Sartorius and Krammer, 2002). In addition to this, overexpression of Bcl-2 and $p21^{Waf1/Cip1}$ in human A549 cells induces resistance to cisplatin (Zhang et al., 1999). In vitro selection with camptothecin generates the human NSCLC cell line $A549^{CPT}$, which is resistant to drug-induced apoptosis by virtue of attenuation of caspase-3-like protease activity, as compared with parental A549 cells (Zhang et al., 1999). Likewise, transfection of either Bcl-2 or $p21^{Waf1/Cip1}$ cDNA into parental A549 cells resulted in resistance to apoptosis, while treatment with Bcl-2 and $p21^{Waf1/Cip1}$ antisense oligodeoxynucleotides restored drug susceptibility in $A549^{CPT}$ cells (Zhang et al., 1999). These results indicate that coinduction of Bcl-2 and $p21^{Waf1/Cip1}$ in $A549^{CPT}$ cells may be involved in acquired drug resistance by inhibition of caspase-mediated apoptosis (Zhang et al., 1999). Long-term exposure of A549 cells to the protein kinase inhibitor 7-hydroxystaurosporine (UCN-01) selected cells ($A549^{UCN}$) with acquired resistance against UCN-01. $A549^{UCN}$ exhibited a 14-fold resistance against cisplatin compared with the parental A549 line, and resistant cells were characterized by overexpression of the CDK inhibitors p21 and p27, and of cyclins D1 and E and of Bcl-2 (Sugiyama et al., 1999). In contrast, cyclin A and B1, pRB, and CDK2 were apparently down-regulated, without changes in CDK4/6. UCN-01 hardly affected the expression of cyclin B1 and induced pRB dephosphorylation in both cell types. In $A549^{UCN}$ cells, but not in the parental line, UCN-01 induced down-regulation of cyclin A and CDK2 activity (Sugiyama et al., 1999). In a clinical study 27 NSCLC patients were treated with concomitant daily low-dose cisplatin and radiotherapy with the aim of investigating whether biological factors related to radiosensitivity and chemosensitivity have prognostic relevance (Van de Vaart et al., 2000). Tumor specimens were analyzed for p53 and Bcl-2 expression, cell proliferation (Ki-67), and the occurrence of apoptosis. In addition to this, cisplatin-DNA adducts in epithelial cells of the oral cavity were assessed immunocytochemically. Univariate and multivariate analyses were performed to assess the association between the biological factors and survival over a median follow-up of 41 months, during which 21 patients (78%) died (Van de Vaart et al., 2000). In a univariate analysis age, tumor stage, and cisplatin-DNA adduct staining were the only factors significantly associated with survival ($P < 0.05$, log-rank test), while p53, Bcl-2, Ki-67, and apoptosis showed no relationship with outcome (Van de Vaart et al., 2000). Multivariate analysis revealed that cisplatin-DNA adduct staining remained an independent prognostic factor (hazard ratio, 0.10), with shorter survival times for patients with low cisplatin-DNA adduct staining (Van de Vaart et al., 2000).

Experimental data on the influence of *c-MYC* expression/amplification on chemotherapeutic activity of platinum compounds in NSCLC are lacking. A few studies have examined the chemosensitivity of SCLC and generally found that amplification of the *c-MYC* gene is often correlated with poor prognosis and advanced, pretreated disease. A cisplatin-resistant SCLC subline, $GLC4^{CDDP}$, contains a *c-MYC* amplification; stable transfection of the cell line with an antisense *c-MYC* causes inhibition of cell proliferation, induces apoptosis, reduces clonogenicity, and slightly increases sensitivity to cisplatin in vitro (Van Waardenburg et al., 1996, 1997). The study of the effect of cisplatin exposure on the degree of *N*-MYC amplification in two SCLC cell lines

(H-69, SBC-4) demonstrated that the *N*-MYC gene was amplified approximately 40- and 60-fold in SBC-4 and H-69 cells, respectively, and these two cell lines were more resistant to cisplatin than nine SCLC cell lines without *N*-MYC amplification (Mizushima et al., 1996). In 107 specimens (38 tumors and 69 tumor cell lines) from 90 patients with SCLC, amplification of one of the *MYC* family genes was found in 3 of 40 (8%) untreated patients as compared to 19 of 67 (28%) treated subjects ($P = 0.01$) (Brennan et al., 1991). The *MYC* family DNA amplification occurred in 17 of 54 (31%) tumor samples from patients treated with cyclophosphamide-based combinations and in 2 of 13 (15%) tissue specimens from patients treated with etoposide/cisplatin ($P = 0.25$). Finally, there were no prominent differences in the frequency of amplification following treatment with various chemotherapy regimens, and *MYC* family DNA amplification was similar in tumors and cancer cell lines obtained from the same patients (Brennan et al., 1991). No data are available on the role of the *MYC* family on carboplatin chemosensitivity.

Overexpression of the tyrosine kinase encoded by the *HER-2/neu* gene, $p185^{HER\text{-}2/neu}$, is a common alteration in NSCLC and has been associated with poor prognosis and drug-resistant phenotype. NSCLC cells that overexpress *HER-2/neu* are less sensitive to cisplatin-induced cytotoxicity in comparison to cells expressing low levels of *HER-2/neu* (You et al., 1998). In agreement with this evidence, the in vitro treatment of lung cancer cells with the tyrosine kinase inhibitor CP127,374 and cisplatin was more potent than cisplatin alone with respect to cell growth inhibition and induction of apoptosis (You et al., 1998). In addition to this, to examine whether the tyrosine kinase activity of $p185^{HER\text{-}2/neu}$ is required for proliferation and resistance to chemotherapeutic drugs, NSCLC cells overexpressing *HER-2/neu* were treated with the tyrosine kinase inhibitor emodin alone and in combination with cisplatin. Experimental results showed that emodin preferentially suppressed the proliferation of *HER-2/neu*-overexpressing NSCLC cells and its combination with cisplatin resulted in synergistic inhibition of lung cancer cell growth (Zhang and Hung, 1996). These results indicate that tyrosine kinase activity is required for the chemoresistant phenotype of *HER-2/neu*-overexpressing NSCLC cells and that tyrosine kinase inhibitors can sensitize these cells to chemotherapeutic drugs, including cisplatin (Zhang and Hung, 1996). These data again support the relevant role of *HER-2/neu* signaling in the regulatory balance among cell proliferation, DNA repair, cell cycle checkpoints, and apoptosis (You et al., 1998). Furthermore, in a panel of 20 NSCLC cell lines established from previously untreated patients, high $p185^{HER\text{-}2/neu}$ expression was correlated with chemoresistance, low *S*-phase fraction, and long doubling times (Tsai et al., 1996b). By contrast, cell lines expressing relatively low levels of $p185^{HER\text{-}2/neu}$ were relatively chemosensitive and had higher *S*-phase fraction and shorter doubling times. Multivariate analysis revealed that the level of $p185^{HER\text{-}2/neu}$ was the only independent predictor for chemoresistance to cisplatin (Tsai et al., 1996b). Although intrinsic resistance is likely to be a multifactorial process, overexpression of $p185^{HER\text{-}2/neu}$ may be an important factor affecting drug sensitivity of NSCLC (Tsai et al., 1996b). An additional reason for reduced cisplatin sensitivity of cancer cells overexpressing *HER-2/neu* may be the influence of these signaling pathways on DNA repair activity, particularly through the nucleotide excision repair (NER) system, which plays a major role in the mechanism for repairing DNA damage by cisplatin. Indeed, the investigation of NER activity after cisplatin-induced DNA lesions in a panel of 16 NSCLC cell lines showed that high NER activity was closely correlated with both cisplatin resistance and high expression of $p185^{HER\text{-}2/neu}$. On the contrary, high levels of EGFR showed very little influence on the relationship between $p185^{HER\text{-}2/neu}$ and cisplatin resistance, suggesting that EGFR may be a secondary factor in modulating the chemoresistance of NSCLC cells when compared with *HER-2/neu* (Tsai et al., 2000). Moreover, the immunohistochemical analysis of HER-4 demonstrated its presence in 25% of NSCLC tissues; no response to gemcitabine-cisplatin was documented in HER-4-positive patients, while an objective response was seen in 11 of 15 (73%) HER-4-negative subjects, thus suggesting that the lack of HER-4 expression significantly favored response to chemotherapy (Merimsky et al., 2001).

No experimental findings are available in the literature on the role of *FHIT* in the chemosensitivity of NSCLC. The only data are obtained in a human lung cancer cell line (SCLC-R1) established from a metastatic lesion of SCLC; in vitro, SCLC-R1 cells are sensitive to cisplatin and carboplatin. The SCLC-R1 line is characterized by a translocation involving chromosome 16 and noticeable deletions in both the *FHIT* region in the short arm of chromosome 3 [del(3)(p14)] and in the telomeric region of the short arm of chromosome 12 [del(12)(p13)] (Gasperi-Campani et al., 1998). No amplifications or rearrangements were documented with respect to *c-MYC*, L-MYC, *N*-MYC, *int-2*, *HER-2/neu*, *RAS*, *c-MOS*, and *hst-1* genes, while wild-type p53, pRB, p21K-ras, and p21H-ras gene products were shown. The neuron-specific enolase (NSE) level was much higher in the cytosol of the cell line than in the serum of the patient, and the cell line also had high expression of chromogranin A and cytokeratin 19 (Gasperi-Campani et al., 1998). Finally, overexpression of the excision repair cross-complementing 1 (*ERCC1*) gene, which is crucial in the repair of cisplatin-DNA adducts, is reported to negatively influence the effectiveness of cisplatin-based therapy and was found to be a predictive factor for survival after cisplatin-gemcitabine administration in advanced NSCLC (Lord et al., 2002).

B. Taxanes

Paclitaxel and docetaxel (Fig. 2) are potent chemotherapeutic agents that interfere with mitotic spindle function to block cells at G_2/M, the most radiosensitive phase of the cell cycle. Utilization of paclitaxel as a radiation sensitizer in vivo to treat aggressive, locally advanced neoplasms resulted in high response rates and acceptable toxicity in patients with NSCLC (King et al., 1999). Recent evidence suggests that paclitaxel is unique in its ability to activate apoptosis in tumor cells with *TP53* mutations. To assess whether *TP53* gene therapy may enhance the effect of chemotherapy given sequentially or concurrently with external beam radiation, the combined effects of adenovirus-mediated wild-type *TP53* gene transfer, chemotherapy, and radiation therapy on lung cancer growth in vitro and in vivo were examined on human NSCLC cell lines A549, NCI-H460, NCI-H322, and NCI-H1299. The combination of these three therapeutic modalities, including paclitaxel, synergistically inhibited tumor cell growth at the 50% and 80% inhibitory effect levels in vitro (Nishizaki et al., 2001). In a mouse model with NCI-H1299 and A549 xenografts, combined treatment synergistically inhibited tumor growth in the absence of any apparent increase in toxicity when compared with other treatments and control groups. These findings suggest that a combination of gene therapy, chemotherapy, and radiation therapy may be an effective strategy for human cancer treatment (Nishizaki et al., 2001). Wild-type *TP53* gene transfer by adenoviral vectors in the human NCI-H157 pulmonary squamous cancer cells and NCI-H1299 large cell carcinoma cells induced a modest increase in the anticancer efficacy of paclitaxel, and the analysis of the interaction between paclitaxel and *TP53* transduction demonstrated an additive effect (Osaki et al., 2000). However, it has been demonstrated that adenoviral transfer of wild-type *TP53* in seven NSCLC cell lines with wild-type, deleted, or point-mutated *TP53* synergistically interacts with DNA-damaging agents in most cell lines by enhancing apoptosis, but displays only additive effects with paclitaxel and docetaxel (Horio et al., 2000). In vitro and in vivo studies suggest that paclitaxel may activate tumor cell apoptosis in the presence of *TP53* mutation and trigger tumor cell death by alternate pathways. To assess whether this finding can be translated into the clinical setting, 30 patients with locally advanced (stage III) NSCLC were treated with paclitaxel/radiotherapy. Mutations in *TP53* were found in 12 of 30 patients (40%). The objective response rate was 75% for patients with tumors with p53 mutations, and 83% for patients with wild-type p53, the difference being not statistically significant. Therefore, in this study p53 mutations do not predict the response of patients with NSCLC to paclitaxel/radiotherapy, at variance with the results obtained with other chemotherapeutic agents and ionizing radiation (Safran et al., 1996). A similar investigation was carried out in 25 patients with metastatic NSCLC treated with single-agent paclitaxel. *TP53* mutations in exons 5 through 8 were found in 8 of 25 patients (32%) and the response rates were 75% for patients with *TP53* mutations and 47% for patients with wild-type *TP53*. The 1-year survival rates for patients with and without *TP53* mutations after treatment with weekly paclitaxel were 63 and 53%, respectively, thus confirming that *TP53* mutations do not adversely affect the response rate to paclitaxel as a single agent in metastatic NSCLC (King et al., 2000). Furthermore, ex vivo chemosensitivity testing showed that *TP53* mutations did not correlate with the activity of paclitaxel/carboplatin on cancer cells from primary NSCLCs (Vogt et al., 2002). These findings are in agreement with the observation that the p53-null human NSCLC NCI-H358 cells are sensitive to paclitaxel and that transfection with wild-type *TP53* slightly reduces the chemosensitivity to the taxane (Ling et al., 2000). Indeed, treatment of NCI-H358 cells with paclitaxel blocks cell-cycle progression at the G_2/M phase and increases cyclin B1 and cdc2 expression. On the contrary, the same treatment slightly arrests the cell cycle at the G_2/M phase and elevates cyclin B1 expression in cells transfected with wild-type *TP53*, which appeared to be blocked in the G_1 phase of the cell cycle (Ling et al., 2000). These findings suggest that transduction of cells with wild-type *TP53* triggers the senescence program, which is responsible, at least in part, for the reduced sensitivity to paclitaxel (Ling et al., 2000). Other studies, however, demonstrated that the infection of an adenoviral vector carrying the wild-type *TP53* into the human lung cancer cell lines, NCI-H1299 (deleted p53), RERF-LC-OK (mutant p53), and A549 (wild-type p53), synergistically increased the sensitivity to several anticancer agents, including docetaxel, regardless of the cellular p53 status (Inoue et al., 2000). Finally, *TP53* gene therapy was examined in 25 patients undergoing first-line chemotherapy for advanced NSCLC, including carboplatin at AUC6 plus paclitaxel 175 mg/m^2 (day 1), or cisplatin 100 mg/m^2 (day 1) plus vinorelbine 25 mg/m^2 (days 1, 8, 15, and 22) in combination with intratumoral injection on day 1 of 7.5×10^{12} particles of SCH 58500, a recombinant adenovirus carrying wild-type *TP53*. No difference was observed between the response rate of lesions treated with *TP53* gene therapy in addition to chemotherapy (52% objective responses) and tumors treated with chemotherapy alone (48% objective responses) (Schuler et al., 2001). Subgroup analysis according to the chemotherapy regimens did not reveal evidence for increased local tumor regressions in response to *TP53* gene therapy in patients receiving carboplatin plus paclitaxel (Schuler et al., 2001).

Inhibition of p21ras activation by FTI-2148, which prevents p21ras conjugation with farnesyl moiety, suppressed the growth of the human lung adenocarcinoma A549 cells in nude mice, and combination therapy of

FTI-2148 with paclitaxel resulted in a greater antitumor efficacy than monotherapy (Sun et al., 1999), thus providing indirect evidence of the possible influence of p21ras biological activation on the response to paclitaxel.

Previous reports have demonstrated that reconstitution of $p16^{INK4a}$ has marked effects on the proliferative capacity of tumor cell lines both in vitro and in vivo, and that $p16^{INK4a}$ expression causes resistance to some chemotherapeutic agents. The transduction of $p16^{INK4a}$ with the recombinant adenovirus Adp16 to the $p16^{INK4a}$-negative, pRB-positive bladder cancer cell line EJ caused a profound inhibition of cell proliferation mediated by arrest in the G_1 phase of the cell cycle. In contrast, the $p16^{INK4a}$-positive, pRB-negative cell line J82 was unaffected by this treatment (Grim et al., 1997). However, when adenovirally mediated $p16^{INK4a}$ replacement was combined with the chemotherapeutic agent paclitaxel, a marked chemoresistance was observed in genetically engineered cells (Grim et al., 1997), an effect likely to be dependent on the reconstitution of effective G_1 checkpoint block and reduction of the fraction of cells progressing to the paclitaxel-sensitive G_2/M phase (Grim et al., 1997). The interaction of genetic profile and mutational status of key tumor-suppressor genes with paclitaxel in combination with other agents commonly used in NSCLC was examined in the human NSCLC cell lines A549 ($p16^{INK4a}$ deleted, p53 wild-type, and pRB wild-type), Calu-1 ($p16^{INK4a}$ deleted, p53 deleted, and pRB wild-type), and NCI-H596 ($p16^{INK4a}$ wild-type, p53 mutated, and pRB deleted) (Edelman et al., 2001). The IC_{50} of paclitaxel was similar on the A549 and NCI-H596 cell lines (1.8 and 1.4 nM), albeit it was higher (6 nM) on the Calu-1 cell line (Edelman et al., 2001). Although the authors did not evaluate the statistical relevance of these findings and their possible link with the genetic pattern of cell lines being used, the data appear to indicate that the loss of two major tumor-suppressor genes, i.e., $p16^{INK4a}$ and p53, may adversely affect the chemosensitivity of cells to paclitaxel, although paclitaxel is not a DNA-damaging agent.

The protective effect of Bcl-2 against apoptosis is lost if the protein is phosphorylated; indeed, Bcl-2 phosphorylation can be induced by agents that affect microtubule depolymerization or prevent microtubule assembly, including taxanes and cryptophycins. The human NCI-H460 NSCLC cells express high levels of Bcl-2 and, after a 4-h exposure to paclitaxel 50 nM, Bcl-2 phosphorylation was demonstrated by Western blot analysis (Lu et al., 2001). In NCI-H460 cells, 90% cell killing was obtained after 24 h of exposure to 20 nM paclitaxel, while in the Bcl-2-negative Calu-6 NSCLC cells the same effect was obtained with a lower drug concentration (11 nM) (Lu et al., 2001). Thus, paclitaxel is an inducer of Bcl-2 phosphorylation and can be active also in cells with Bcl-2 overexpression, although the concentrations required to exert its effect are higher in Bcl-2-negative than in Bcl-2-positive cells (Lu et al., 2001). The in vivo efficacy of paclitaxel 60 mg/kg i.v. every 3 weeks was characterized in heterotransplanted human NSCLC tumors characterized with respect to MRP, *HER-2/neu*, EGFR, Bax, and Bcl-2, and p53 (Perez-Soler et al., 2000). The response rate to paclitaxel was 21%, a percentage similar to that reported in phase II studies in patients with advanced NSCLC and treated with single-agent paclitaxel. Tumor parameters significantly associated with response were *HER-2/neu* and Bcl-2 expression; in particular, all responding tumors were *HER-2/neu* (−) and Bcl-2 (+), while 48% of nonresponding tumors were *HER-2/neu* (+) and 43% were Bcl-2 (+) (Perez-Soler et al., 2000). There was a trend toward a higher response rate in Bax-positive tumors and MRP- and EGFR-negative tumors, but it was not statistically significant. The response rate was independent of baseline p53 status and mitotic index. Responding tumors had a higher Bax/Bcl-2 ratio 24 h after therapy, but the difference was only marginally significant (2.8 for responding tumors versus. 1.1 for nonresponding tumors, $P = 0.07$) (Perez-Soler et al., 2000). The combination of two microtubule-active agents, docetaxel and vinorelbine, is able to inactivate the proto-oncogene Bcl-2 through protein phosphorylation. Indeed, the administration of docetaxel (60 mg/m^2) and vinorelbine (45 mg/m^2) every 2 weeks to chemotherapy-naive patients with advanced NSCLC was associated with a major objective response rate of 51%, a median survival time of 14 months, and 1-year survival rate of 60%, suggesting that this drug combination is highly active for the treatment of advanced NSCLC (Miller et al., 2000).

The cytotoxic activity of paclitaxel is dependent on the interaction with microtubules, which are heterodimers of α- and β-tubulin; both of them are guanosine triphosphate (GTP)-binding proteins and β-tubulin is a GTPase, whereas α-tubulin has no enzyme activity. Binding sites for paclitaxel have been demonstrated on the β-tubulin subunit that has six isotypes. The increased expression of the brain-specific human class III β-tubulin isotype, encoded by the *Hβ4* gene, is associated with paclitaxel resistance in ovarian tumors and NSCLC cell lines (Kavallaris et al., 1999). The treatment of paclitaxel-resistant A549-T24 NSCLC cells, which display a 4-fold increase in *Hβ4* expression compared to parental A549 cells, with 1 μM antisense oligodeoxynucleotides targeted against various regions of the *Hβ4* gene reduced mRNA expression by 40 to 50%, and the abundance of the class III β-tubulin isotype corresponded to a 39% increase in sensitivity to paclitaxel. These findings support the role of *Hβ4* and class III β-tubulin expression in paclitaxel resistance and have potential implications for the treatment of paclitaxel-resistant tumors (Kavallaris et al., 1999). An additional mechanism of paclitaxel resistance might involve mutations in GTP- and paclitaxel-binding domains of β-tubulin in tumor cells. β-Tubulin mutations in exons 1 and 4 were ob-

served in the DNA isolated from biopsy specimens of 16 patients (33%) of a cohort of 49 subjects with advanced or metastatic NSCLC (Monzo et al., 1999). Patients were treated with two schedules of paclitaxel, 210 mg/m^2 over 3 h and 200 mg/m^2 over 24 h, and none of the patients with β-tubulin mutations had an objective response, whereas 39.4% of patients without β-tubulin mutations had complete or partial responses (Monzo et al., 1999). Median survival was 3 months for the 16 patients with β-tubulin mutations and 10 months for the 33 patients without β-tubulin mutations, the difference being highly statistically significant (Monzo et al., 1999). The analysis of 20 lung cancer cell lines and 22 specimens from NSCLC patients showed silent mutations at codon 180 of the β-tubulin gene, which encodes the GTP-binding site of the protein, and at codons 195 and 217 (Tsurutani et al., 2002). However, neither missense nor nonsense mutations within or near the GTP-binding site of the β-tubulin gene were detected. These results indicate that β-tubulin gene mutations might not play a major role in the mechanism of resistance to paclitaxel in this selected patient population (Tsurutani et al., 2002).

Acquired resistance to paclitaxel can be mediated by several mechanisms, including overexpression of P-gp, altered expression of β-tubulin isotypes, intrinsic or acquired mutations in β-tubulin, and expression of novel genes. β-Tubulin mutations were recently identified in 33% of 49 NSCLC patients, none of whom had an objective response to paclitaxel treatment (Rosell and Felip, 2001). Cisplatin resistance is associated with several molecular alterations, including overexpression of metallothionein and the mRNA level of the excision repair cross-complementing (*ERCC1*) gene. Early detection of circulating cancer cells in peripheral blood and the analysis of DNA abnormalities may be used to monitor the effects of therapy. In particular, serum DNA can be used as a surrogate for detecting genetic abnormalities and as a potential guide for customizing treatment. Indeed, β-tubulin mutations in serum DNA were detected in 42% of 131 NSCLC patients and in none of the healthy individuals (Rosell and Felip, 2001).

Overexpression of P-gp by tumors results in multidrug resistance to structurally unrelated chemotherapeutic agents including vinca alkaloids, anthracyclines, taxanes, and epipodophyllotoxins, and this type of resistance may be reverted by P-gp antagonists; one such agent, LY335979, fully restored sensitivity to paclitaxel in CEM/VLB100 leukemia cells at 0.1 μM (Dantzig et al., 1996). LY335979 blocked [^{3}H]azidopine photoaffinity labeling of P-gp in CEM/VLB100 plasma membranes and competitively inhibited equilibrium binding of [^{3}H]vinblastine to P-gp, with a K_i value of approximately 0.06 μM. Furthermore, the inhibition of P-gp activity enhanced the antitumor activity of paclitaxel in a model of a human NSCLC nude mouse xenograft (Dantzig et al., 1996). On the basis of the crucial importance of P-gp in modulating the therapeutic response to paclitaxel, in vivo imaging of P-gp has been attempted to assess the likelihood of response of tumors to P-gp-transportable drugs, including paclitaxel. For this purpose, 99mTc-tetrofosmin was administered to 20 patients with stage III or IV NSCLC before chemotherapy with paclitaxel and tumor-to-normal lung ratios, and retention indices were calculated by chest imaging to assess the expression of P-gp in NSCLC (Kao et al., 2001). The early and delayed mean tumor-to-normal lung ratios were 1.59 ± 0.25 and 1.50 ± 0.25, respectively, for 10 patients with a good response to paclitaxel and 1.09 ± 0.09 and 1.03 ± 0.05, respectively, for 10 patients with a poor response ($P < 0.001$). On the contrary, retention index was not a predictive factor of response to paclitaxel as other prognostic factors, including age, sex, tumor size, stage, and cell type. Therefore, in vivo imaging of P-gp may be useful in predicting the chemotherapeutic response to paclitaxel (Kao et al., 2001; Shiau et al., 2001).

C. Gemcitabine

Gemcitabine (Fig. 2) is a cytidine analog that is intracellularly phosphorylated by deoxycytidine kinase to gain cytotoxic activity that is dependent on inhibition of ribonucleotide reductase and drug incorporation into the DNA during the *S* phase. Deoxycytidine kinase is a rate-limiting enzyme required for the activation of the pyrimidine analog cytarabine (Beausejour et al., 2002), the most widely used agent for the chemotherapy of hematological malignancies. Deoxycytidine kinase also plays an important role in the activation of several new agents for the treatment of leukemia, such as the purine analogs 2-chloro-deoxyadenosine and fludarabine. Gemcitabine has remarkable therapeutic activity as a single agent against several solid malignancies, such as NSCLC, suggesting that deoxycytidine kinase is a widely distributed important target for the activation of antimetabolites in solid tumors. Studies on the regulation of deoxycytidine kinase have shown that the enzyme has a complex regulation since it undergoes feedback inhibition by intracellular nucleotides (Singhal et al., 1992). In addition to this, there is an inverse relationship between doubling time and deoxycytidine kinase activity in cancer cell lines, with rapidly growing cells (doubling time of approximately 20 h) showing the highest activity, and slower-growing cells (doubling time of approximately 60 h) showing the lowest enzyme activity (Singhal et al., 1992). In the human NSCLC cell line SW1573, 5 μM cortisol and 100 nM dexamethasone decreased the sensitivity of cancer cells to gemcitabine, cortisol reduced deoxycytidine kinase activity in SW1573 cells, while dexamethasone decreased, in the same cells, the activity of thymidine kinase 2, an enzyme involved in the salvage pathway of gemcitabine (Bergman et al., 2001b). These data provide evidence that the interference of drugs on metabolic pathways of gemcitabine might be clinically relevant, particularly with steroids such as dexamethasone, which are frequently used

to treat side effects of cytotoxic therapy (Bergman et al., 2001b). Furthermore, a high level of resistance to gemcitabine in human lung carcinoma SW-1573 cells is associated with deoxycytidine kinase deficiency at mRNA and protein levels; sensitivity to other antitumor drugs was not altered, except for cytarabine (van Bree et al., 2002). Cytosolic 5′-nucleotidase is responsible for deactivation of nucleotides; solid tumors, such as adenocarcinomas of the lung, are frequently hypoxic and are, therefore, likely to exhibit increased nucleotide breakdown through the 5′-nucleotidase pathway (Blay et al., 1997). 5′-Nucleotidase activity is likely to play an important role in tumor resistance to nucleoside analogs; although this hypothesis has not been investigated in lung cancer, data are presented in the literature demonstrating that increased enzyme activity is likely to be responsible for the decreased amount of ribonucleotides and deoxyribonucleotides in leukemia cells resistant to 2-chloro-deoxyadenosine and cytarabine (Lotfi et al., 2001). Cytidine deaminase irreversibly inactivates gemcitabine to the metabolite difluoro-deoxyuridine (Beausejour et al., 2002); the enzyme is widely distributed within tissues and its activity was found to be high in tissues from various types of human cancers, including NSCLC (Miwa et al., 1998). Indeed, gemcitabine was less effective against tumors xenotransplanted to animals with high levels of cytidine deaminase in cancer cells (Miwa et al., 1998). Of note, NCI-H69^{DAU}, a daunorubicin-resistant variant of NCI-H69 with overexpression of P-gp, and NYH^{VM}, a teniposide-resistant variant of NYH with an altered topoisomerase II target, were more sensitive to gemcitabine than the parental cell lines because of a 4.3- and 2-fold increased activity of deoxycytidine kinase, respectively (Bergman et al., 2001a). Furthermore, cytidine deaminase was 9-fold lower in NCI-H69^{DAU} cells (Bergman et al., 2001a). However, recent results obtained in the human A549 NSCLC cells transduced with deoxycytidine kinase demonstrated that difluoro-deoxyuridine was cytotoxic to the A549^{dCK} cells, but not to the wild-type cells, possibly as a result of the activity of the mitochondrial thymidine kinase, an important modulator of gemcitabine-induced cell toxicity (Beausejour et al., 2002).

To explore the level of expression of genes encoding enzymes of nucleoside metabolism, a recent study provided evidence of heterogeneous expression of deoxycytidine kinase, 5′-nucleotidase, and cytidine deaminase in tumor tissue from 42 untreated patients with NSCLC (De Braud et al., 2001), suggesting the possible use of gene expression profiling of drug-metabolizing enzymes to predict chemosensitivity to gemcitabine (Fig. 3). Gemcitabine and paclitaxel are active agents in the treatment of NSCLC; their simultaneous or sequential combination in the NSCLC cell lines NCI-H460, NCI-H322, and Lewis Lung resulted in comparable cytotoxicity, varying from additivity to antagonism. Gemcitabine caused an S (48%) and G_1 (64%) arrest at IC_{50} and

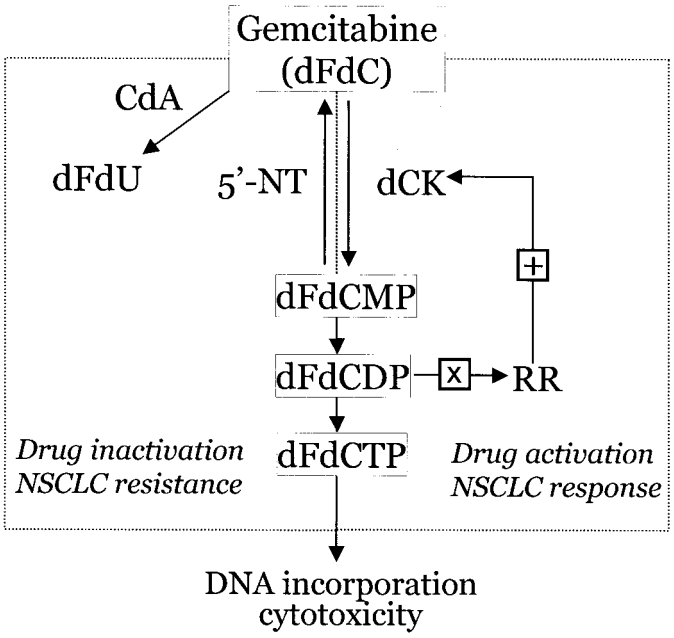

FIG 3. Intracellular metabolism of gemcitabine (dFdC) to difluorodeoxyuridine (dFdU), and to mono- (dFdCMP), di- (dFdCDP) and triphosphate (dFdCTP) metabolites. Phosphorylation of gemcitabine by deoxycytidine kinase (dCK), inhibition of ribonucleotide reductase (RR) by dFdCDP and incorporation of dFdCTP into DNA are associated with cytotoxicity and tumor response. On the contrary, deamination of gemcitabine by cytidine deaminase (CdA) to produce dFdU and dFdCMP dephosphorylation by 5′-nucleotidase is associated with drug inactivation and tumor resistance. Deoxycytidine kinase may be up-regulated as a consequence of ribonucleotide reductase inhibition; in addition to this, DNA repair may induce dCK activity.

10-fold the IC_{50}, respectively (Kroep et al., 2000). Paclitaxel induced G_2/M arrest (70%) which was maximal within 24 h at 10-fold the IC_{50}. Apoptosis was more pronounced when paclitaxel preceded gemcitabine, as compared to the reverse sequence. In NCI-H460 cells, paclitaxel increased the accumulation of the active triphosphate metabolite of gemcitabine 2-fold in contrast to NCI-H322 cells (Kroep et al., 2000). Paclitaxel did not affect deoxycytidine kinase, but nucleotide levels increased, possibly explaining the high concentrations of the active triphosphate metabolite of gemcitabine reached within cells; moreover, gemcitabine incorporation into the RNA was enhanced (Kroep et al., 2000). Gemcitabine almost completely suppressed DNA synthesis in cell lines (70–89%), while paclitaxel had a minor effect and did not enhance the inhibitory effect of gemcitabine on DNA synthesis (Kroep et al., 2000). Therefore, gemcitabine-paclitaxel did not show sequence-dependent cytotoxic effects and all combinations were not more than additive. However, since paclitaxel increased active triphosphate metabolite accumulation, gemcitabine incorporation into RNA, and apoptotic index, the administration of paclitaxel before gemcitabine may be preferred compared to the reverse sequence (Kroep et al., 2000). Indeed, a clinical study in which the sequence of paclitaxel followed by gemcitabine was administered on a weekly basis to chemotherapy-naive

patients with stage III-IV NSCLC demonstrated objective responses at all dose levels, with an overall response rate of 43% in 30 evaluable patients (De Pas et al., 2000). Hematological toxicity included grade 4 neutropenia, grade 3 thrombocytopenia, and febrile neutropenia. The worst nonhematological toxicity was grade 3 elevation in serum transaminases and grade 2 neurosensory toxicity in 8% and 5% of cycles, respectively. This study demonstrated that the weekly administration of paclitaxel and gemcitabine is well tolerated, and has promising antitumor activity in NSCLC at the recommended dose of 100 mg/m^2 paclitaxel and 1500 mg/m^2 gemcitabine (De Pas et al., 2000).

Gemcitabine induces apoptosis in drug-sensitive cells; therefore, the involvement of Bcl-2 superfamily members was investigated. Previous studies have demonstrated that several splice variants are derived from both the caspase 9 and *Bcl-x* genes in which the *Bcl-x* splice variant, *Bcl-xL*, and the caspase 9 splice variant, caspase 9b, inhibit apoptosis in contrast to the pro-apoptotic splice variants, *Bcl-xS* and caspase 9 (Chalfant et al., 2002). Treatment of A549 NSCLC cells with gemcitabine down-regulated the levels of anti-apoptotic *Bcl-xL* and caspase 9b mRNA with a concomitant increase in the mRNA levels of pro-apoptotic *Bcl-xS* and caspase 9 (Chalfant et al., 2002). In addition to this, the Fas/FasL system in lung cancer cells was examined upon exposure to gemcitabine. All lung cancer cell lines (NCI-H460, NCI-H322, GLC4, GLC4/ADR, NCI-H187, and N417) expressed Fas and FasL at RNA and protein levels, and apoptosis could be induced in 4 of 6 cell lines upon exposure to the Fas agonist monoclonal antibody CLB-CD95/15 (Ferreira et al., 2000b). After gemcitabine exposure no significant FasL upregulation was observed, whereas Fas expression was increased in the wild-type p53 cell line NCI-H460, but not in cells with mutant p53 (Ferreira et al., 2000b). Moreover, no correlation was observed in lung cancer cell lines between sensitivity to gemcitabine and to the antibody CLB-CD95/15; in addition to this, preincubation of cells with either the Fas-antagonist antibody CLB-CD95/2 or a FasL-neutralizing antibody did not protect from drug-induced apoptosis. Finally, caspase-8 activation was observed upon drug exposure independently from Fas/FasL signaling. Taken together, these observations provide evidence against a role of the Fas/FasL signaling pathway in drug-induced apoptosis in lung cancer cells (Ferreira et al., 2000b).

The effects of gemcitabine on expression of p53 and p21, cell proliferation and induction of apoptosis, and cell cycle distribution were evaluated in the human lung cancer cell lines NCI-H460 and NCI-H322. Gemcitabine inhibited cell growth and induced apoptosis in a concentration- and time-dependent manner; flow-cytometry analysis of DNA at 4, 24, 48, and 72 h after treatment with gemcitabine at IC_{80} demonstrated the accumulation of cells in the G_1 phase (Tolis et al., 1999). Gemcitabine induced p53 and p21 expression in the p53 wild-type NCI-H460 cell line but not in the p53 mutant NCI-H322 cell line; the percentage of cells expressing p53 was highest after treatment with drug concentrations corresponding to IC_{80}, whereas the highest percentage of p21-positive cells could be induced by treatment with gemcitabine at IC_{50} (Tolis et al., 1999). These findings suggest that low concentrations of gemcitabine induce cell cycle arrest through the activity of p53 and p21, whereas higher drug concentrations induce p53-mediated apoptosis (Tolis et al., 1999), thus providing evidence of the importance of wild-type p53 expression in the chemosensitivity to gemcitabine. To evaluate whether the major tumor-suppressor genes are involved in the response of NSCLC cells in vitro to gemcitabine, the human cell lines A549 ($p16^{INK4a}$ deleted, p53 wild-type, and pRB wild-type), Calu-1 ($p16^{INK4a}$ deleted, p53 deleted, and pRB wild-type), and NCI-H596 ($p16^{INK4a}$ wild-type, p53 mutated, and pRB deleted) were evaluated with respect to gemcitabine alone and in combination with carboplatin and paclitaxel (Edelman et al., 2001). The IC_{50} of gemcitabine was similar in the A549 and NCI-H596 cell lines (11 and 10 nM), albeit somewhat higher (18 nM) on the Calu-1 cell line (Edelman et al., 2001), again indicating that the integrity of at least $p16^{INK4a}$ or p53 tumor-suppressor genes renders cells more sensitive to growth inhibition by the nucleoside analog. In the A549 cell line all combinations of gemcitabine with carboplatin and paclitaxel demonstrated antagonism at lower fractions affected and synergism at higher fractions affected. On the contrary, the NCI-H596 cell line displayed the highest sensitivity to all drug combinations, while in Calu-1, bearing a deletion in both $p16^{INK4a}$ and p53, the simultaneous administration of gemcitabine and carboplatin was borderline additive or antagonistic (Edelman et al., 2001). Overall, $p16^{INK4a}$ and p53 appear important determinants of chemosensitivity of cancer cells to gemcitabine.

Inhibition of p21ras activation by FTI-2148 suppressed the growth of the human lung adenocarcinoma A-549 cells in nude mice and combination therapy of FTI-2148 with gemcitabine resulted in a more enhanced antitumor efficacy than monotherapy (Sun et al., 1999), thus suggesting a potential negative role of p21ras activation by mutation or overexpression on the activity of gemcitabine. Furthermore, transfection of the NCI-H460 human NSCLC cell line with the anti-apoptotic genes Bcl-2 or Bcl-xL was associated with increased clonogenic survival of cells treated with gemcitabine from 15% to 50–54%, thus indicating that the cytotoxic effect of gemcitabine is antagonized by Bcl-2 and Bcl-xL (Ferreira et al., 2000a).

The intrinsic resistance of lung cancer cells against gemcitabine does not correlate with the level of *HER-2/neu* expression, although a modest reduction in the chemosensitivity to the nucleoside analog is seen in cells with high $p185^{HER\text{-}2/neu}$ levels (Tsai et al., 1996a). In

addition to this, the combination of gemcitabine with DNA-damaging agents, including cisplatin, may be active against NSCLC cells overexpressing *HER-2/neu*, because $p185^{HER\text{-}2/neu}$ up-regulation is associated with a more effective DNA repair ability, thus attenuating the lethal effects of chemotherapeutic agents. Furthermore, the comparison of the interaction among gemcitabine-cisplatin, gemcitabine-etoposide, and cisplatin-etoposide in a panel of 12 NSCLC cell lines and the analysis of the correlations between the level of $p185^{HER\text{-}2/neu}$ and drug cytotoxicity demonstrated little cross-resistance of gemcitabine to either etoposide or cisplatin (Tsai et al., 1996a). Furthermore, gemcitabine-containing combinations demonstrated equivalent or superior activity compared to cisplatin-etoposide, with gemcitabine-cisplatin showing a greater synergistic activity (Tsai et al., 1996a). The effect of cisplatin-etoposide was not related to $p185^{HER\text{-}2/neu}$ expression, whereas gemcitabine-containing regimens, especially gemcitabine-cisplatin, had a greater cytotoxicity against cells with high levels of $p185^{HER\text{-}2/neu}$ (Tsai et al., 1996a). These findings indicate that the gemcitabine-cisplatin combination is more active than etoposide-cisplatin, particularly in cells with enhanced expression of the *HER-2/neu* gene (Tsai et al., 1996a).

D. Epipodophyllotoxins

Epipodophyllotoxins are tight-binding inhibitors of topoisomerases II, and etoposide (Fig. 2) is the epipodophyllotoxin most widely used in NSCLC. A study examined the effect of introducing a mutant *TP53* gene into the NCI-H460 cell line, which carries a wild-type *TP53* gene, and the wild-type *TP53* gene into the NCI-H1437, NCI-H727, NCI-H441, and NCI-H1299 cells carrying a p53 protein mutated at amino acid residues 143, 175, 248, and 273, respectively, on the chemosensitivity to several anticancer agents, including etoposide (Lai et al., 2000). The representative cell line NCI-H1437 cells transfected with wild-type *TP53* genes showed a dramatic increase in the susceptibility to etoposide compared to untransfected NCI-H1437. An increase in chemosensitivity to etoposide was also observed in wild-type *TP53* transfectants of NCI-H727, NCI-H441, and NCI-H1299 cells (Lai et al., 2000). In contrast, loss of cytotoxicity by etoposide and a lack of p53-mediated cell death were observed in NCI-H460 cells transfected with mutant *TP53*. These observations suggest that *TP53* gene status modulates the degree of chemosensitivity and induction of apoptosis by etoposide in NSCLC cells (Lai et al., 2000). Using a panel of 7 NSCLC cell lines with wild-type, deleted, or point-mutated *TP53*, the in vitro cytotoxicity of etoposide was examined in combination treatment with a *TP53* transduction by an adenoviral vector. Gene transduction and etoposide showed synergistic effects in 6 of 7 cell lines and additive effects against a p53-mutated cell line (Horio et al., 2000). Flow cytometry and DNA fragmentation analysis revealed that *TP53* transduction enhanced the apoptotic death induced by etoposide in 6 of 7 cell lines (Horio et al., 2000). These results suggest that wild-type *TP53* plays an important role in cell response to etoposide and gene transduction may synergistically enhance the chemosensitivity of the majority of NSCLC cells to DNA-damaging agents due to the enhancement of apoptosis (Horio et al., 2000). Additional studies provided evidence that *TP53* transduction by an adenoviral vector into the NSCLC cell lines NCI-H1299 (p53 deleted), RERF-LC-OK (p53 mutated), and A549 (wild-type p53) increased the sensitivity to etoposide regardless of the baseline *TP53* status, and a synergism between gene-therapy and etoposide was confirmed (Inoue et al., 2000). The immunohistochemical analysis of p53 in 146 surgically resected specimens of NSCLC demonstrated that 65 of 146 samples (45%) showed abnormal p53 protein accumulation in >10% of cancer cells within the tumor tissue (p53+), whereas 81 (55%) were p53−, in which no or less than 10% positive cancer cells were detected (Higashiyama et al., 1998). In vitro chemosensitivity testing on surgical samples to several anticancer agents, including etoposide, was then performed by a collagen gel-droplet embedded culture system. Although it was demonstrated that p53-negative tumors were significantly more sensitive to some anticancer agents, including 5-fluorouracil, than p53-positive samples, the same finding was not observed with etoposide (Higashiyama et al., 1998). To investigate whether *TP53* affects the expression of MRP, one of the major factors for non-P-gp-mediated multidrug resistance in lung cancer, 107 NSCLCs were examined by immunohistochemistry and it was shown that 43.9% were positive for MRP (Oshika et al., 1998). In addition to this, NSCLC specimens with mutant p53 showed a significant correlation with MRP upregulation and coexpression of MRP and p53 in the same NSCLC cells was also demonstrated (Oshika et al., 1998). Twenty-six patients with MRP-positive tumors who underwent postoperative chemotherapy with MRP-transportable anticancer drugs, such as etoposide, had significantly poorer prognosis than did those with MRP-negative tumors (Oshika et al., 1998). In addition to this, NSCLC patients with coexpression of MRP and p53 showed poorer clinical course than did those without MRP and p53 ($P = 0.014$). These results suggest that MRP overexpression is affected by mutant p53 and this combination adversely influences the prognosis of NSCLC (Oshika et al., 1998). Finally, etoposide treatment of *TP53*-mutated SCLC H69 and GLC4 cell lines is not associated with the increase in the p53-target gene $p21^{Waf1/Cip1}$; thus the progression of cells through the G_2/M phase is allowed and apoptosis may occur (Liu et al., 2002).

Activation of the HER-2/neu gene is frequently encountered in NSCLCs and has been linked to shortened survival. The analysis of chemosensitivity of 20 NSCLC cell lines established from untreated patients demon-

strated that mutation of the TP53 gene was a common event (18 of 20 lines); there was no relationship, however, between mutations at any specific codon and chemoresistance. On the contrary, multivariate analysis revealed that the expression of HER-2/neu was the only independent predictor for chemoresistance to etoposide (Tsai et al., 1996b). Further investigation of the role of the HER-2/neu-encoded tyrosine kinase $p185^{HER\text{-}2/neu}$ on the chemosensitivity and drug-induced cell cycle changes of NSCLC cell lines demonstrated that high-$p185^{HER\text{-}2/neu}$-expressing cells were more resistant to etoposide, but displayed enhanced chemosensitivity to tyrphostin AG825, a preferential inhibitor of $p185^{HER\text{-}2/neu}$, which may be used in combination chemotherapy to sensitize tumor cells to the activity of etoposide (Tsai et al., 1996c). A study on the effect of HER-2/neu overexpression and *RAS* point mutations on the chemosensitivity to several anticancer agents, including etoposide, on 20 NSCLC cell lines established from untreated patients was in agreement with other studies on this issue and demonstrated a significant direct correlation between the IC_{50} values for all drugs and the degree of HER-2/neu gene expression in all 20 NSCLC cell lines (Tsai et al., 1993). The IC_{50} values for etoposide in cells with RAS mutations were slightly lower than in those without RAS mutations (borderline significance, $P = 0.031$). Interestingly, HER-2/neu expression in cell lines with RAS mutations was lower than in those without RAS mutations, although the difference was not significant. These findings confirm that overexpression of HER-2/neu is a marker for intrinsic multidrug resistance and of therapeutic failure in NSCLC (Tsai et al., 1993).

The relationships between chemoresistance and the presence of RAS point mutations in 20 NSCLC cell lines established from untreated patients demonstrated that RAS mutations do not adversely affect the activity of etoposide in NSCLC (Tsai et al., 1993). According to this finding, H-ras transduction in the NCI-H82 SCLC cells (NCI-H82Hras) resulted in a phenotypic change toward NSCLC with no change in etoposide sensitivity (Kaufmann et al., 1995). This observation was further confirmed by a study in patients affected by NSCLC; subjects were treated with chemotherapy including mesna, ifosfamide, carboplatin, and etoposide, and stratification on the basis of K-RAS mutational status showed that the response rate and progression-free and overall survival were not affected by K-RAS mutation in the primary tumor (Rodenhuis et al., 1997).

The analysis of the relationship between pRB expression levels and cytotoxicity of etoposide in a panel of NSCLC cell lines with wild-type pRB provided evidence of a significant direct correlation between chemosensitivity and high levels of pRB expression ($P = 0.049$) (Yamamoto et al., 1998). The analysis of the effect of etoposide on pRB status and cell cycle distribution of the NSCLC cell lines Ma-12, the most sensitive, and Ma-31, the most resistant, showed that etoposide, after a 24-h exposure at 0.1–10 μM, suppressed pRB expression and induced pRB dephosphorylation and accumulation of Ma-12 cells in the G_2/M phase of the cell cycle (Yamamoto et al., 1998). On the contrary, etoposide exposure was associated with phosphorylation of pRB and no changes in pRB expression and cell cycle distribution in Ma-31 cells (Yamamoto et al., 1998). Despite these in vitro findings, the analysis of pRB expression in clinical specimens was not correlated with clinical outcome. Indeed, absent or aberrant pRB expression was detected in 12 of 80 NSCLC specimens (Shimizu et al., 1994). A stable, hypophosphorylated mutant pRB was detected in three NSCLC samples. Analysis of the matched clinical data showed no associations between pRB status and age, sex, extent of disease, performance status, smoking history, and previous treatment (Shimizu et al., 1994). Retrospective analysis showed no correlation of pRB expression with best clinical response, overall survival, or in vitro chemotherapeutic drug sensitivity (Shimizu et al., 1994). An investigation was carried out to study the relationship between the immunocytochemical expression of topoisomerases IIα and IIβ, MRP, p53, p21, and Bcl-2 in primary tumor samples from 93 patients who were then treated with etoposide-based chemotherapeutic regimens (Dingemans et al., 1999). High levels of topoisomerases IIα and IIβ were associated with shorter survival and lower complete response rate, respectively. In addition to this, multivariate analysis demonstrated that high expression of topoisomerase IIα and Bcl-2 were predictive for shorter survival (Dingemans et al., 1999). Therefore, high expression of topoisomerases IIα and IIβ and Bcl-2 adversely affects the efficacy of etoposide-based chemotherapy (Dingemans et al., 1999). No data are reported in the literature on the influence of *c-MYC* on chemoresponsiveness to etoposide. The available data are obtained in SCLC, in which a dysregulation of both *c-MYC* gene expression and retinoid signaling pathways commonly occurs. Preclinical data provide evidence that all-*trans*-retinoic acid inhibits SCLC in vitro growth and affects *c-MYC* expression (Kalemkerian et al., 1998). The administration of combination chemotherapy with etoposide (120 mg/m^2 i.v. on days 1–3), cisplatin (60 mg/m^2 i.v. on day 1), and all-*trans*-retinoic acid (150 mg/m^2/day p.o. for up to 1 year) in patients with SCLC induced 1 complete response and 9 partial responses in 22 assessable patients, for an overall response rate of 45% (Kalemkerian et al., 1998). The median survival was 10.9 months and the 1-year survival was 41%, being similar to treatment with cisplatin and etoposide alone (Kalemkerian et al., 1998). These data indirectly argue against a major role of *c-MYC* expression in tumor cell sensitivity to etoposide. To characterize the mechanisms associated with chemoresistance in NSCLC, the expression of P-gp, MRP, and LRP was examined using the A549 cell line selected for resistance to etoposide. The wild-type A549

cells strongly express LRP, while the MRP protein is expressed at a moderate level and P-gp is not detected (Trussardi et al., 1998). Induction of resistance to etoposide paralleled an increase in the expression of the *MRP* gene and a decrease in *LRP*. These results indicate that NSCLC cells exhibit a complex pattern of drug-resistance proteins, still susceptible to evolve under treatment (Trussardi et al., 1998). In an additional study, *MRP* gene expression was examined by reverse transcriptase-polymerase chain reaction (RT-PCR) and protein immunoblotting in 15 unselected cell lines, and it was found that MRP was frequently expressed in NSCLC, with markedly varying intensity (Berger et al., 1997). Two cell lines expressed high MRP levels without amplification of the *MRP* gene. Using daunomycin as MRP substrate and verapamil as MRP modulator, transporting activity of MRP was related to its gene expression (Berger et al., 1997). Moreover, a significant correlation between *MRP* expression and chemoresistance against a number of anticancer agents, including etoposide, was observed (Berger et al., 1997). A clear relationship between *MRP* gene expression and sensitivity to etoposide was also observed in vitro in nine lung cancer cell lines not expressing the *MDR1* gene. In one cell line, drug resistance was related with high expression of DNA topoisomerase II (Narasaki et al., 1997). Furthermore, a positive correlation between *MRP* gene expression in three cell lines and the modulatory effect of verapamil on etoposide was demonstrated. These data were not confirmed in SCLC cells, thus suggesting that MRP is likely to be involved in intrinsic multidrug resistance in NSCLC rather than in SCLC (Narasaki et al., 1997). As a matter of fact, NSCLC and SCLC differ in their clinical response to topoisomerase II-directed drugs, such as etoposide and teniposide, as NSCLC is virtually insensitive to single-agent therapy, while SCLC responds in two-thirds of cases (Kreisholt et al., 1998). Preclinical studies demonstrated that resistance to topoisomerase IIα inhibitors depends on enzyme content and activity as well as on P-gp and MRP. Immunocytochemical analysis of untreated tumor tissue from 27 cases of NSCLC and 29 cases of SCLC demonstrated that NSCLC had significantly less topoisomerase IIα than SCLC, as only 5 of 27 NSCLC cases had more than 5% positive cells compared with 28 of 29 SCLC, and 0 of 27 NSCLC had more than 25% positive cells compared with 26 of 29 SCLC tissue specimens (Kreisholt et al., 1998). P-gp was detected in more than 5% of cells in only 3 of 27 NSCLC and 6 of 29 SCLC, and MRP in 5 of 27 NSCLC and 9 of 29 SCLC. After treatment of patients with either etoposide or teniposide there was a significant increase in MRP and P-gp expression in SCLC, while topoisomerase IIα decreased (Kreisholt et al., 1998). In conclusion, the major difference between NSCLC and SCLC was in topoisomerase IIα content, which is in turn related to the clinical activity of etoposide (Kreisholt et al., 1998).

E. Vinca Alkaloids

Vinorelbine is a semisynthetic vinca alkaloid in which the catharanthine moiety contains an eight-membered ring in place of the nine-membered ring that is present in all naturally occurring compounds of the vinblastine group (Fig. 2). This modification selectively reduces the interaction with anoxal versus mitotic microtubules and may account for the lower neurotoxicity with improved antitumor activity observed in clinical trials in patients with NSCLC. Vinorelbine leads to phosphorylation of Bcl-2 on serine residues, leading to inactivation of this protein and facilitating the unopposed action of the proapoptotic protein Bax (Haldar et al., 1997). This mechanism of action is also possessed by docetaxel; indeed, preclinical in vitro data on the reciprocal enhancement of chemotherapeutic activity of vinorelbine and docetaxel provided evidence that the combination of two microtubule-active agents is active (Aoe et al., 1999; Zoli et al., 1999) due to the distinct drug targets on tubulin, yet converging on Bcl-2/Bax balance that ultimately results in the triggering of apoptosis (Wang et al., 1999). These findings are in agreement with the results of a clinical trial on the combination of vinorelbine 45 mg/m^2 by i.v. bolus injection followed by docetaxel 60 mg/m^2 as a 1-h i.v. infusion given every 2 weeks in 35 chemotherapy-naive patients with advanced NSCLC (Miller et al., 2000). The objective response rate was 51%; with a median follow-up of 14 months, the predicted median survival time was 14 months, and the 1-year survival rate was 60%. Febrile neutropenia occurred in five patients and symptomatic onycholysis and excessive lacrimation were observed after several months of therapy, while no dose-limiting neurotoxicity occurred. Thus, the docetaxel and vinorelbine combination is an active regimen for the treatment of advanced NSCLC (Miller et al., 2000). In vitro chemosensitivity testing by a collagen gel-droplet embedded culture system on 146 surgically resected specimens of NSCLC, with 45% of them showing abnormal p53 accumulation in $\geq$10% of cancer cells (p53+) and 55% showing $<$10% positive cancer cells (p53−), demonstrated no relationship between p53 protein status and in vitro chemosensitivity to the vinca alkaloid vindesine (Higashiyama et al., 1998). This result is not unexpected in view of the effect of vinca alkaloids on the cell cycle, which is dependent on the interference with mitotic spindle function to block cells at the G_2/M phase; therefore, cell death occurs in a p53-independent way. The investigation of the clinical efficacy of combination chemotherapy with p53 gene therapy has nonetheless attracted great interest for the role of p53 in the cell death induced by other antitumor drugs, including alkylating agents. For these reasons, studies aimed at restoring normal p53 function by means of adenovirus-expressing wild-type p53 in tumors with deleted or mutated p53 have been carried out. In a phase II study gene therapy was complemented with

simultaneous cisplatin and vinorelbine treatment (Boulay et al., 2000). The efficiency of gene transduction was monitored by biopsies of neoplastic tissue obtained from all treated patients before and 24 to 48 h after gene therapy. In most of the cases the p53 target gene $p21^{Waf1/Cip1}$ was upregulated, especially when the injection of higher doses of p53-expressing adenovirus was combined with simultaneous chemotherapy (Boulay et al., 2000). Interestingly, a clear $p21^{Waf1/Cip1}$ gene response was observed only in tumors showing stabilization or regression. Hence, $p21^{Waf1/Cip1}$ appears to be upregulated after adenovirus-mediated p53 gene transfer, and it is apparently the most sensitive marker tested for biological response to gene therapy in NSCLC (Boulay et al., 2000). However, the benefit of p53 gene therapy has yet to be fully demonstrated because a recent phase II study on 25 patients with unresectable NSCLC failed to provide a clear additional benefit from intratumoral p53 delivery compared to the effect that can be achieved by effective first-line chemotherapy. Indeed, patients received three cycles of carboplatin (AUC 6; day 1) plus paclitaxel (175 mg/m^2, day 1), or cisplatin (100 mg/m^2, day 1) plus vinorelbine (25 mg/m^2, days 1, 8, 15, and 22) in combination with intratumoral injection on day 1 of 7.5×10^{12} particles of the recombinant adenoviral vector SCH 58500 carrying a wild-type p53 (Schuler et al., 2001). There was no difference between the response rate of lesions treated with p53 gene therapy in addition to chemotherapy (52% objective responses) and lesions treated with chemotherapy alone (48% objective responses) (Schuler et al., 2001). Subgroup analysis according to the chemotherapy regimens revealed evidence for increased mean local tumor regressions in response to additional p53 gene therapy in patients receiving cisplatin-vinorelbine, but not in patients receiving carboplatin-paclitaxel (Schuler et al., 2001). There was no survival difference between the two chemotherapy regimens, and the median survival of the cohort was 10.5 months (1-year survival, 44%). Efficient p53 transduction was confirmed in tumor samples from 68% of patients, and toxicities attributable to gene therapy were mild to moderate (Schuler et al., 2001). Possible explanations to the findings of this study are the following: 1) the enhancement of cytotoxic activity following p53 transduction is expected from the alkylating agents cisplatin and carboplatin, not from drugs, such as paclitaxel and vinorelbine, acting on the p53-independent G_2/M phase of the cell cycle; 2) efficient wild-type p53 transduction may reduce the fraction of cells that progress to the G_2/M phase of the cell cycle, which is sensitive to microtubule-active anticancer drugs; and 3) adenoviral infection of deep portions of the tumor mass may be negligible, thus preventing efficient *TP53* gene delivery.

A possible advantage in restoring normal p53 function may be explained by the correlation observed between p53 and MRP in NSCLC. Indeed, the analysis of 107 NSCLCs for MRP and p53 expression by immunohistochemistry demonstrated that 43.9% specimens were positive for MRP, and tumor tissues with mutant p53 showed a significant correlation with MRP overexpression (Oshika et al., 1998). Twenty-six patients with MRP-positive tumors who underwent postoperative chemotherapy with MRP-related anticancer drugs, including vindesine, had significantly poorer prognoses than did those with MRP-negative tumors ($P = 0.017$). This correlation between MRP expression and prognosis was also seen in stage III patients ($P = 0.022$) and in patients with squamous cell carcinoma ($P = 0.062$) (Oshika et al., 1998). NSCLC patients with coexpression of MRP and p53 showed poorer prognoses than did those without MRP and p53 ($P = 0.014$) (Oshika et al., 1998). These results suggest that MRP overexpression affected by mutant p53 has a significant effect on prognosis through non-P-gp-mediated multidrug resistance in NSCLC (Oshika et al., 1998).

In addition to the mechanism of drug resistance to vinca alkaloids, including vinorelbine, vindesine, vincristine, and vinblastine, mediated by MRP, P-gp also represents an important drug transport system that plays a relevant role in the limitation of cytotoxic activity of vinca alkaloids. Despite the chemical modification of vinorelbine, the drug is a P-gp substrate and displays cross-resistance with other drugs transported by P-gp. Indeed, vinorelbine-resistant, murine P388 leukemia cells were cross-resistant to P-gp-transportable drugs, including vinblastine and vincristine, but not to the alkylating agents cyclophosphamide, carmustine, and cisplatin or to the antimetabolites 5-fluorouracil and methotrexate (Adams and Knick, 1995). Cellular resistance to vinorelbine was stable without drug exposure during continuous passage in vivo for more than 10 weeks and in vitro for at least 5 weeks. P388-resistant cells exhibited increased expression of P-gp and a 30-fold level of resistance of vinorelbine in vitro, which was completely reversible with verapamil (Adams and Knick, 1995). Therefore, enhanced expression of the *MDR1* gene renders cells cross-resistant to several cytotoxic agents and antagonizes the antitumor effect of vinca alkaloids, including vinorelbine. For these reasons, P-gp antagonists have been developed to revert the drug-resistant phenotype to drug sensitivity. First-generation P-gp antagonists were characterized by low potency and occurrence of adverse effects (i.e., verapamil); on the contrary, the more recent compounds showed enhanced activity and ability to chemosensitize cells to several cytotoxic agents. In particular, GF120918, at 250 ng/ml, increased the sensitivity of a multidrug-resistant SCLC cell line (H69/LX4) to the P-gp substrates paclitaxel, taxotere, vinblastine, vinorelbine, and etoposide to levels that were either greater (in the case of etoposide) or close to that of the parent cell line (Myer et al., 1999). This was achieved despite the remarkable variation in the levels of resistance of the cell line for

various anticancer drugs tested, and even in the case of high levels of resistance, as was the case for paclitaxel and taxotere (Myer et al., 1999).

No data are available on the effect of *c-MYC* expression on the chemotherapeutic activity of vinorelbine. The inhibition of *c-MYC* gene expression by an inducible antisense expression vector in the SCLC cell line GLC4cDDP, containing *c-MYC* gene amplification and resistant to cisplatin, did not result in modification in the pattern of cell chemosensitivity to vincristine, thus suggesting a limited impact of *c-MYC* gene function on cytotoxicity by vinca alkaloids (Van Waardenburg et al., 1997).

F. Ifosfamide and Cyclophosphamide

Ifosfamide (Fig. 2) and cyclophosphamide are alkylating agents widely used for the treatment of solid tumors and hematological malignancies including NSCLC, breast cancer, and lymphomas. Although studies addressing the influence of *MYC* amplification on the chemotherapeutic effect of ifosfamide or cyclophosphamide are not available in the literature, a study of 90 patients with SCLC demonstrated that amplification of one of the *MYC* family genes was detected in 3 of 40 (8%) untreated patient specimens compared to 19 of 67 (28%) samples from treated subjects, the difference being statistically significant ($P = 0.01$) (Brennan et al., 1991). In addition to this, the amplification of *MYC* DNA copy number occurred in 17 of 54 (31%) of the specimens from patients treated with chemotherapeutic combinations including cyclophosphamide (Brennan et al., 1991). These results demonstrate that *MYC* family DNA amplification occurs more commonly in specimens from treated than untreated patients, thus suggesting that chemotherapy exerts a selective pressure to the development of cells with amplified *MYC* genes.

Ex vivo chemosensitivity of human NSCLC obtained from 28 cases of primary tumors showed that *TP53* mutations were associated with resistance to cyclophosphamide-etoposide-epirubicin (Vogt et al., 2002). Furthermore, wild-type *TP53* gene transfer by adenoviral vectors in the human NCI-H157 squamous cell lung carcinoma, and the human NCI-H1299 large cell lung cancer, induced a modest increase in the anticancer efficacy of cyclophosphamide in vitro and the analysis of interaction demonstrated an additive effect between cyclophosphamide and wild-type *TP53* transduction (Osaki et al., 2000).

Mutated *K-RAS* constitutes an adverse prognostic factor in stage I or II lung cancer and has been linked to resistance to ionizing radiations and some therapeutic agents. For these reasons, its inclusion in the staging system of lung cancer, together with TNM classification, has been proposed (Rosell et al., 1995). To determine whether the clinical course and the response to chemotherapy of patients with advanced adenocarcinoma of the lung depends on the presence or absence of a *K-RAS* mutation, patients with advanced adenocarcinoma of the lung were treated with ifosfamide, carboplatin, and etoposide, and genotypical analysis of their tumor samples was performed to demonstrate the presence of *K-RAS* gene mutations (Rodenhuis et al., 1997). *K-RAS* mutations could be established in 69 of 83 patients (83%); chemotherapy with ifosfamide, carboplatin, and etoposide was administered to 62 patients, 16 of whom were shown to have a *K-RAS* mutation-positive tumor (Rodenhuis et al., 1997). The patterns of metastases, response, and survival were similar between the *K-RAS* mutation-positive and *K-RAS* mutation-negative groups (Rodenhuis et al., 1997). Therefore, patients with advanced lung adenocarcinoma who harbor a *K-RAS* mutation may have major responses to chemotherapy and have similar progression-free and overall survival as patients with *K-RAS* mutation-negative tumors (Rodenhuis et al., 1997). At variance with these data, a study in 40 patients with stage III NSCLC who underwent tumor resection after neoadjuvant treatment with ifosfamide, carboplatin, and etoposide and subsequent radio-chemotherapy with carboplatin and vindesine demonstrated that *K-RAS* codon 12-point mutations were found in 13 of 28 resection specimens (46%) and, even after complete resection, the presence of a *K-RAS* mutation was a significant predictor for a poor progression-free survival (Broermann et al., 2002).

G. Novel Agents

1. Topoisomerase I Inhibitors. Topotecan and irinotecan (Fig. 4) are semisynthetic derivatives of camptothecin with potent topoisomerase I inhibitory activity and a wide range of antitumor efficacy in vitro and in vivo. Topotecan and irinotecan, like other DNA-damaging agents, arrests or delays cell cycle progression during S and G_2 phases in a wide variety of tumor-derived cell lines. Particularly, the G_2 arrest gives time for the cell to repair DNA lesions before starting a new cell cycle. For these reasons, the control of these restriction points plays a relevant role in the modulation of anticancer effect by topoisomerase I inhibitors. A study examined the effect of the introduction of mutant *TP53* gene into the NCI-H460 cell line, which carries a wild-type *TP53* gene, and the introduction of wild-type *TP53* gene into NCI-H1437, NCI-H727, NCI-H441, and NCI-H1299 cells, which carry a p53 mutated at amino acid residues 143, 175, 248, and 273, respectively (Lai et al., 2000). The representative cell line NCI-H1437 cells transfected with wild-type *TP53* gene showed a dramatic increase in the susceptibility to camptothecin. An increase in chemosensitivity was also observed in wild-type *TP53* transfectants of NCI-H727, NCI-H441, and NCI-H1299 cells (Lai et al., 2000). In contrast, loss of chemosensitivity and a lack of p53-mediated DNA degradation in response to anticancer agents were observed in NCI-H460 cells transfected with mutant *TP53*. These observations suggest that *TP53* gene status modulates

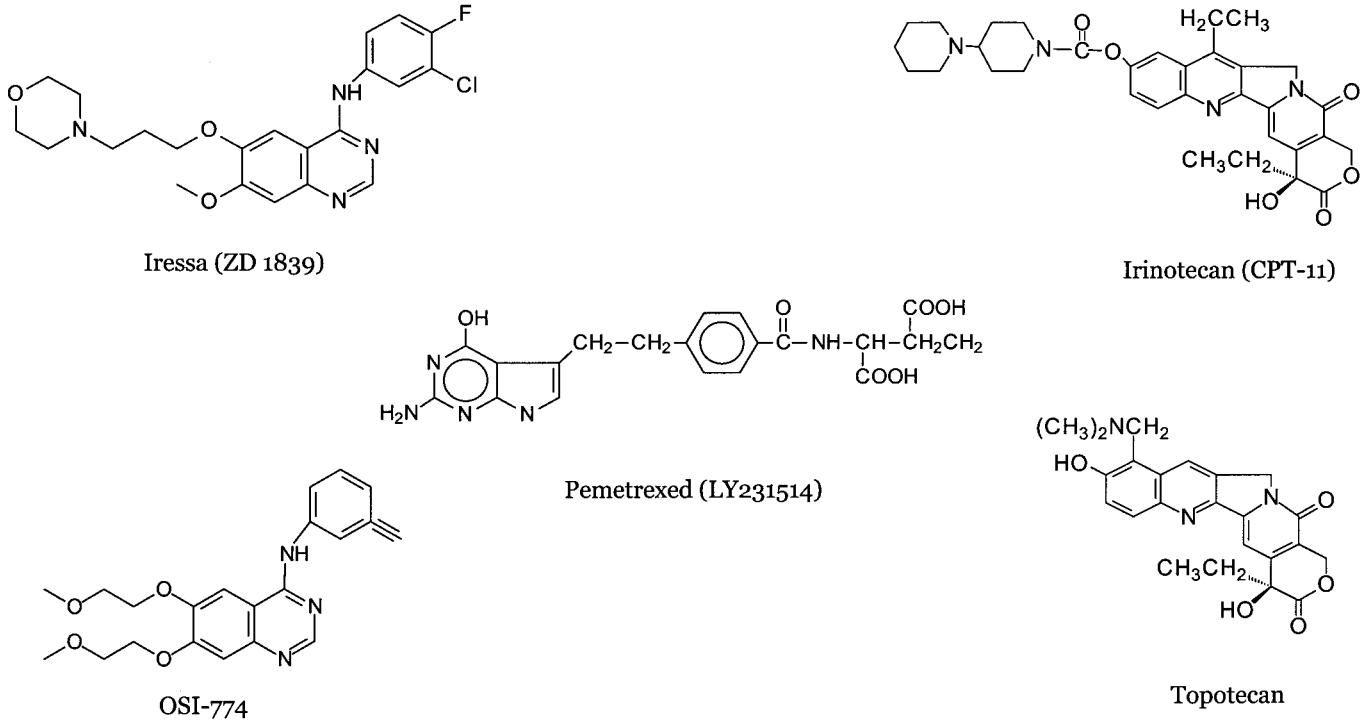

FIG 4. Chemical structures of investigational agents for the treatment of NSCLC.

the extent of chemosensitivity and the induction of apoptosis by different anticancer agents in NSCLC cells, including the topoisomerase I inhibitor camptothecin (Lai et al., 2000). Wild-type *TP53* gene transfer in a human pulmonary squamous cell carcinoma, the NCI-H157 cell line, and a human pulmonary large cell carcinoma, the NCI-H1299 cell line, enhanced in a supra-additive manner the effectiveness of 7-ethyl-10-hydroxy-camptothecin (SN-38), the active metabolite of irinotecan on NCI-H157 cells, while an additive effect was observed on NCI-H1299 cells (Osaki et al., 2000). These findings suggest that irinotecan may thus be useful as a possible anticancer agent in a combination therapy regimen using wild-type *TP53* gene transfer (Osaki et al., 2000). In line with these observations, the combined treatment of human lung cancer cell lines, NCI-H1299 (deleted p53), RERF-LC-OK (mutant p53), and A549 (wild-type p53), with anticancer drugs, including irinotecan, and a recombinant adenoviral vector expressing wild-type *TP53* cDNA, increased the sensitivity to the drug, regardless of the cellular *TP53* status, and a synergism was observed by the isobologram analysis (Inoue et al., 2000). The study of the interaction between topotecan and the G_2/M-active agent etoposide in the *TP53*-mutated NCI-H23 NSCLC cell line demonstrated that short-term sequential topotecan→etoposide treatment was effective in inducing cytotoxicity in cancer cells (Taron et al., 2000). Detailed analysis of results evidenced that: 1) sequential topotecan→etoposide was synergistic when drug administration overlapped the maximum percentage of topotecan-induced G_2/M phase cell arrest interval; and 2) the reverse sequential schedule (etoposide→topotecan) was only additive, thus providing evidence of sequence-dependent effects of anticancer treatment (Taron et al., 2000). Using a panel of 7 NSCLC cell lines with wild-type (2 cell lines) or abnormal (2 null and 3 point-mutated cell lines) *TP53*, it was demonstrated that the in vitro combined effects of the DNA-damaging agent SN-38 and the adenoviral vector carrying wild-type *TP53* was synergistic with respect to cytotoxicity on six of seven cell lines and additive against a p53-mutated cell line (Horio et al., 2000). Flow cytometric and DNA fragmentation analyses revealed that a sublethal dose of *TP53*-adenoviral vector augmented the apoptotic response induced by SN-38 in six of seven cell lines, thus demonstrating that wild-type *TP53* transduction may enhance the chemosensitivity of NSCLCs to DNA-damaging agents (Horio et al., 2000). Topotecan displays potent growth inhibitory activity on the human lung cancer cell lines NCI-H460 (wild-type p53) and NCI-H322 (mutant p53). Induction of apoptosis after treatment is concentration- and time-dependent, and treatment with topotecan at IC_{80} was most effective in triggering DNA fragmentation (Tolis et al., 1999). DNA analysis by flow-cytometry indicates that topotecan at IC_{80} causes accumulation of cells in S and G_2/M phases and induces the expression of both p53 and p53-related target $p21^{Waf1/Cip1}$ in the NCI-H460 cell line but not in NCI-H322, which carries a mutant p53 (Tolis et al., 1999). The percentage of cells expressing p53 is highest at IC_{80} values, whereas the highest percentage of $p21^{Waf1/Cip1}$ positive cells could be induced by in vitro

treatment at IC_{50} values, suggesting that p53 induces cell cycle arrest at low drug concentrations and apoptosis at higher concentrations (Tolis et al., 1999). These findings provide evidence of the important role of p53 and related targets, including $p21^{Waf1/Cip1}$, on the response of NSCLC cells to topoisomerase I inhibitors.

Preliminary findings indicate that *CDKN2A* ($p16^{INK4a}$) enhanced the cytotoxicity of topoisomerase I inhibitors in NSCLC cells (Nishio et al., 1997). Indeed, gene transduction provided evidence that the sensitivity of $p16^{INK4a}$-transfected A549 cells was increased by up to 9.1-fold with respect to irinotecan and SN-38, as compared with parental, $p16^{INK4a}$ deleted A549 cells (Fukuoka et al., 1997). Furthermore, the topoisomerase I-mediated DNA relaxation activity in $p16^{INK4a}$-transduced cells was approximately five times higher than those of the parental A549 cells (Fukuoka et al., 1997). RNA and protein analyses indicated that increased topoisomerase I activity of $p16^{INK4}$-transduced cells was dependent on increased enzyme mRNA and protein levels (Fukuoka et al., 1997). Thus, $p16^{INK4a}$ transduction is able to restore the chemosensitivity to topoisomerase I inhibitors by means of the increment in topoisomerase I mRNA level, protein content, and activity in cells previously lacking functional $p16^{INK4a}$ (Fukuoka et al., 1997). Further analysis of the effect of $p16^{INK4a}$ transduction on the in vitro chemosensitivity of A549 NSCLC cells to the topoisomerase I inhibitor irinotecan showed that transfected cells were more prone to undergo apoptosis with respect to $p16^{INK4a}$-deleted parental A549 (Fukuoka et al., 2000). Apoptosis was suppressed by inhibitors of interleukin-1β-converting enzyme (ICE/caspase-1) or ICE-like proteases, as determined by DNA fragmentation and proteolytic cleavage of poly(ADP-ribose) polymerase, a natural substrate for caspase-3 (Fukuoka et al., 2000). In *CDKN2A*-transfected A549 cells, cytosolic peptidase activities increased during irinotecan-induced apoptosis and were suppressed by the highly specific caspase-3 and caspase-3-like inhibitor, Z-DEVD-fluoromethyl-ketone (Fukuoka et al., 2000). These findings indicate that $p16^{INK4a}$ is involved in the activation pathway of caspase-3 induced by irinotecan, in addition to its regulatory activity on topoisomerase I levels (Fukuoka et al., 2000). Furthermore, a delay in *S*-phase progression and induction of apoptosis were observed after treatment with irinotecan in *CDKN2A*-transduced A549 cells, which also displayed down-regulation of cyclin A, whereas cyclin B, cdk2, and cdc2 protein levels were unaffected (Fukuoka et al., 2000).

Recent findings demonstrate that overexpression of Bcl-2 and $p21^{Waf1/Cip1}$ in the human A549 NSCLC cell line induces resistance to apoptosis to a variety of anticancer drugs, including camptothecin (Zhang et al., 1999). The multidrug-resistant cell line $A549^{CPT}$ was established by in vitro selection with camptothecin, and $A549^{CPT}$ cells were resistant to drug-induced apoptosis because of remarkable attenuation of caspase-3-like protease activity compared with parental A549 cells (Zhang et al., 1999). The main mechanisms associated with resistance to apoptosis in $A549^{CPT}$ cells were overexpression of anti-apoptotic Bcl-2 and $p21^{Waf1/Cip1}$ (Zhang et al., 1999). Transfection of either *Bcl-2* or $p21^{Waf1/Cip1}$ cDNA into parental A549 cells resulted in resistance to apoptosis, and the cotreatment of $p21^{Waf1/Cip1}$ and *Bcl-2* antisense oligodeoxynucleotides restored drug susceptibility in $A549^{CPT}$ cells more effectively than either one of them alone (Zhang et al., 1999). These results indicate that coinduction of *Bcl-2* and $p21^{Waf1/Cip1}$ in $A549^{CPT}$ cells may be involved in acquired drug resistance against anticancer agents, such as topoisomerase I poisons, by inhibiting caspase-mediated apoptosis (Zhang et al., 1999). In addition to this, transfection of the NCI-H460 human NSCLC cell line with the anti-apoptotic genes *Bcl-2* or *Bcl-xL* is associated with increased clonogenic survival of cells treated with topotecan, from 16% to 45–46%, thus confirming that the cytotoxic effect of topotecan is antagonized by *Bcl-2* and *Bcl-xL* (Ferreira et al., 2000a). Finally, resistance to irinotecan has been observed in a *Bcl-2*-transfected human SCLC cell line, $SBC\text{-}3^{Bcl\text{-}2}$, as compared with the parental line SBC-3, while there was no difference in sensitivity to cisplatin and paclitaxel between SBC-3 and $SBC\text{-}3^{Bcl\text{-}2}$ (Ohmori et al., 1993).

The overexpression of the *MDR1* gene encoding P-gp is associated with a drug-resistant phenotype against topotecan, as shown in KB-V1 cells, selected for vinblastine resistance, and in NIH3T3 cells, transfected with a plasmid expressing P-gp (NIH-MDR-$G_1$85) (Hoki et al., 1997). However, NIH-MDR-$G_1$85 cells displayed no significant resistance to other topoisomerase I inhibitors, such as 9-aminocamptothecin, camptothecin, and SN-38, while KB-V1 cells were cross-resistant to these compounds (Hoki et al., 1997). The expression of the drug transport systems P-gp and MRP1 and 2 do not seem to play a relevant role in drug resistance to camptothecin and SN-38 (Cummings et al., 2002b); therefore, additional mechanisms, including enhanced clearance by glucuronidation from cancer cells (Cummings et al., 2002a), mutation of the topoisomerase I gene (Urasaki et al., 2001), and reduced levels of topoisomerase I, resulting in decreased enzyme activity (Pommier et al., 1999) are likely to be implicated. The clinical relevance of these findings remains to be demonstrated, however, since topotecan does not apparently induce P-gp expression, and multidrug-resistant tumors are usually not resistant to topotecan (Houghton et al., 1995). The study of the cytotoxic effects of SN-38 on multidrug-resistant human glioblastoma GB-1 cells and nondrug-resistant human glioblastoma U87-MG cells demonstrated that SN-38 is significantly more active against GB-1 and U-87MG cells than camptothecin (Nakatsu et al., 1997). Furthermore, DNA analysis showed that SN-38 induced apoptosis in these tumors, thus suggesting that SN-38 has significant cytotoxic activity on malignant glioma

cells regardless of the expression of drug-resistant mechanisms (Nakatsu et al., 1997). Additional studies on the role of P-gp in drug resistance against topoisomerase I inhibitors demonstrated that camptothecin produced equivalent amounts of cell growth inhibition and/or DNA single-strand breaks in the drug-sensitive (AuxB1) and multidrug-resistant (CHRC5) Chinese hamster ovary cells, while topotecan, SN-38, and 9-aminocamptothecin were up to 12-fold less toxic to the multidrug-resistant cells than to the wild-type cells (Mattern et al., 1993). These findings are consistent with differences in yields of DNA single-strand breaks produced in AuxB1 and CHRC5 cells by treatments with the various compounds (Mattern et al., 1993). The resistance ratios of the topoisomerase I inhibitors were approximately one-tenth those of drugs known to be substrates of P-gp; thus, cells that overexpress P-gp have the potential to develop some level of cross-resistance to the topoisomerase I inhibitors (Mattern et al., 1993). Nonetheless, topotecan has a substantial therapeutic effect on B6D2F1 mice implanted with multidrug-resistant sublines of P388 leukemia compared with its effect on mice implanted with wild-type P388 cells (Mattern et al., 1993).

Limited data are available in the literature concerning the role of the activated *RAS* oncogene in cellular responses to topoisomerase I inhibitors. The transduction of the viral *H-RAS* oncogene in NCI-H82 human SCLC was associated with a 2-fold increase in topoisomerase I activity and a 1.7-fold decrease in the IC_{50} for camptothecin (Kaufmann et al., 1995). These changes resulted in 30-fold lower survival of NCI-H82^{rasH} cells compared with parental NCI-H82 cells at camptothecin concentrations as low as 10 nM (Kaufmann et al., 1995). Although these data were obtained by gene transduction in a SCLC cell line, it may be speculated that in NSCLC cells bearing an isolated *RAS* mutation the increase in topoisomerase I associated to DNA may facilitate the occurrence of lethal strand breaks by topoisomerase I inhibitors and cell death.

2. Epidermal Growth Factor Receptor Inhibitors. ZD1839 (Iressa), OSI-774 (Fig. 4), and C225 (cetuximab) are agents that target the interaction between EGFR (ErbB-1) and EGF or block signal transduction mediated by EGF (Ciardiello and Tortora, 2001). The critical role of the EGF autocrine loop in cancer progression has led to an extensive search for selective inhibitors of the EGFR signaling pathway. The results of a large body of preclinical studies and the early clinical trials thus far conducted suggest that targeting the EGFR could represent a significant contribution to cancer therapy (Ciardiello and Tortora, 2001). A variety of different approaches are currently being used to inhibit cell signaling through EGFR. The most promising strategies in clinical development include monoclonal antibodies to prevent EGF binding and small molecule inhibitors of the tyrosine kinase activity that inhibit autophosphorylation and downstream intracellular signaling (Ciardiello and Tortora, 2001). At least five blocking monoclonal antibodies have been developed against the EGFR (ErbB-1). Among these, IMC-C225 (Cetuximab) is a chimeric human-mouse monoclonal IgG_1 antibody that has been the first anti-EGFR targeted therapy to enter clinical evaluation in cancer patients, alone or in combination with radiotherapy or chemotherapy. A number of small molecule inhibitors of the EGFR tyrosine kinase enzymatic activity are also in development. Preclinical and clinical findings indicate that ZD1839 (Iressa) and OSI-774 are active against a broad spectrum of tumors, particularly NSCLC, and have an acceptable safety profile.

The comparison of mRNA expression of *EGFR*, *HER-2/neu*, and survival in primary tumors and matching nonmalignant tissues from 83 patients with NSCLC demonstrated that *EGFR* and *HER-2/neu* mRNAs were detectable in all specimens analyzed (Brabender et al., 2001). Twenty-nine (34.9%) and 28 (33.7%) patients had high *HER-2/neu* and *EGFR* expression, respectively. High HER-2/neu and *EGFR* coexpression were detectable in 14 (16.9%) patients and were associated with shorter survival ($P = 0.004$), whereas high *EGFR* expression showed only a trend toward inferior survival ($P = 0.176$) (Brabender et al., 2001). The impact of *HER-2/neu* and *EGFR* coexpression on patients' survival was additive ($P = 0.003$), and multivariate analysis determined high *HER-2/neu* expression ($P = 0.041$) and high *EGFR/HER-2/neu* coexpression ($P = 0.030$) as significant and independent unfavorable prognostic factors (Brabender et al., 2001). These findings indicate that HER-2/neu and EGFR play a crucial role in the biological behavior of NSCLC, and the high prevalence of tumors with elevated EGFR expression justify the targeting of the EGFR signal transduction to treat NSCLC (Brabender et al., 2001). To study the prognostic value of the expression of *EGFR* (ErbB-1), *HER-2/neu* (ErbB-2), and p53, and the amplification of *EGFR*, *HER-2/neu*, and *HER-3* (ErbB-3) in NSCLC, tumor tissue specimens from 118 patients with NSCLC were examined (Reinmuth et al., 2000). Most NSCLC tissue specimens analyzed were aneuploid (81%), 14% showed positive staining for EGFR, 18% for p185$^{HER-2/neu}$, and 41% for p53. There were normal mean gene copy numbers in 86% for *EGFR*, 94% for *HER-2/neu*, and in 96% for *HER-3*. No significant correlations were noted among EGFR, p185$^{HER-2/neu}$, and p53 expression, ploidy status and tumor stage (Reinmuth et al., 2000). The molecular analysis of 65 samples from normal lung and tumor tissue demonstrated that lung tumors with abnormalities of *TP53* at exons 4–8 had a significantly higher EGFR content ($P < 0.001$), *S* phase fraction ($P < 0.007$), DNA index ($P < 0.017$), and a lower proportion of cells in G_1/G_0 phase ($P < 0.04$) than lung tumors with no such *TP53* gene mutations (Lopez-Guerrero et al., 1998). In normal lung the cellular growth fraction decreases significantly with the intensity of p53 expression, and the

lack of biologically functional p53 is apparently related to fast-growing lung cancers (Lopez-Guerrero et al., 1998). To determine the combined effect of growth factor receptor and tumor-suppressor gene abnormalities on the prognosis of NSCLC, 290 tumor specimens comprising 155 cases of stage I, 30 cases of stage II, 96 cases of stage III, and 9 cases of stage IV were examined by immunohistochemical staining on formalin-fixed, paraffin-embedded materials to detect EGFR and p53 in tumor tissue (Ohsaki et al., 2000). Histological subtypes included 142 adenocarcinomas, 127 squamous cell carcinomas, 17 large cell carcinomas, and 4 other types of malignancy (Ohsaki et al., 2000). Positive staining for EGFR was demonstrated in 124 (42.8%) cases; more EGFR-positive cases were found in squamous cell carcinomas than in nonsquamous cell carcinomas ($P = 0.0121$). Staining for p53 protein was observed in 147 (50.7%) specimens (Ohsaki et al., 2000). Multivariate proportional hazard modeling of data revealed that EGFR protein expression was a risk factor ($P = 0.0240$); patients negative for both EGFR and p53 survived for a longer period of time ($P = 0.0427$) (Ohsaki et al., 2000). Although the relationship between target expression and therapeutic efficacy of EGFR inhibitors has not been examined in these studies, it may be speculated that the high incidence of tumors overexpressing EGFR is the prerequisite for activity of inhibitors and that p53 abnormalities should not be relevant for the activity of such inhibitors, since their mechanism of action does not involve DNA damage and does not require the activation of a p53-dependent apoptotic pathway to induce cell death.

The study of the expression of *ErbB-1*, *K-RAS* gene mutations and *c-MYC* gene amplification was performed in tumor and normal lung tissues from 100 patients with NSCLC. The amount of EGFR in 97 tumor and 82 normal tissues was 16.0 ± 3.7 and 10.8 ± 2.0 fmol/mg, respectively (mean ± S.D., $P = 0.015$) (Sekine et al., 1998). Overexpression of the EGF receptor was observed in 6 (24.0%) of 25 squamous cell carcinomas, 16 (23.2%) of 69 adenocarcinomas, and 23 (23.7%) of a total of 97 tumors. *K-RAS* mutations were observed in 9 of 100 tumors; of these, 5 (55.6%) mutations were in codon 12, 1 (11.1%) was in codon 22, and 3 (33.3%) were in codon 61 (Sekine et al., 1998). The patterns of the mutations were GC transversions in three (33.3%) tumors, GC transitions in two (22.2%), and AT transversions in four (44.4%) tumors. There was no association between *EGFR* overexpression and *K-RAS* mutation; c-*MYC* amplification was studied in 23 neoplastic tissues but was not detected (mean number of copies = 1.28 ± 0.24) (Sekine et al., 1998). Of note, the expression of *c-MYC* is influenced strongly by the presence of growth factors, such as EGF (Niklinski et al., 2001). For these reasons, the mutual interaction between the *c-MYC* oncogene and EGF might be disrupted by the use of EGFR inhibitors. The oncogenes *c-MYC* and *TGF-α* are frequently overexpressed in human bronchiolo-alveolar adenocarcinomas, and experiments in transgenic mice demonstrate that *c-MYC* and EGF are directly involved and cooperate with one another during formation of bronchiolo-alveolar adenocarcinomas in the lung (Ehrhardt et al., 2001), thus providing additional evidence on the role of *c-MYC* and growth factors in NSCLC tumorigenesis. In addition to this, Southern blot analysis revealed coamplification of *ErbB-1* (7p12) and *c-MYC* (8q24) (Taguchi et al., 1997). In a group of 81 human squamous cell carcinomas of the lung, overexpression of ErbB-1 oncoprotein (EGFR) was detected in 79% of the tumors, *HER-2/neu* in 35%, and *c-MYC* in 48%; patients with ErbB-1 positive tumors had a poor prognosis (Volm et al., 1992). In addition, these tumors were more frequently drug-resistant; therefore, EGFRs may serve as prognostic factors for the aggressiveness of squamous cell carcinomas of the lung and for the response of these tumors to chemotherapy (Volm et al., 1992).

3. Folic Acid Analogs. The pyrrolopyrimidine-based antifolate pemetrexed (LY231514, Fig. 4) is a multitargeted folate analog in which a pyrrole ring replaces the pyrazine moiety of the pterine portion of folic acid and a methylene group replaces the benzylic nitrogen in the bridging portion of the molecule (Mendelsohn et al., 1999). While this compound inhibits thymidylate synthase, it also inhibits other folate-dependent enzymes including dihydrofolate reductase, aminoimidazole carboxamide ribonucleotide formyltransferase, and glycinamide ribonucleotide formyltransferase (Mendelsohn et al., 1999). Like other folic acid analogs, it is a substrate of folylpolyglutamate synthase; the drug is efficiently metabolized to highly polyglutamated active species by this enzyme and utilizes the reduced folate carrier for entry into the cell to exert its antitumor activity (Mendelsohn et al., 1999). Pemetrexed has shown a broad spectrum of anticancer activity, including NSCLC in humans (Rusthoven et al., 1999). The administration of pemetrexed 600 mg/m^2 every 3 weeks to 59 chemotherapy-naive patients with surgically incurable NSCLC was associated with an overall response rate of 15.8%; the median duration of response was 4.9 months, and the median survival was 7.2 months. The principal toxicities were myelosuppression and rash (Clarke et al., 2002).

The analysis of cytotoxicity by novel folic acid antagonists on the NCI-H69/P SCLC cell line and its multidrug-resistant subline NCI-H69/LX4, which overexpresses *MDR1*, as well as on COR-L23/P large cell line and its subline COR-L23/R overexpressing *MRP*, demonstrated that H69/LX4 and COR-L23/R sublines both showed clear cross-resistance to a number of folic acid antagonists associated with a marked reduction in cellular drug accumulation (Robson et al., 1998). Verapamil, as a drug-resistance modifier, showed little activity, whereas cyclosporin A was more active and partially restored drug accumulation in H69/LX4 overexpressing

P-gp. Hence, novel antifolates appear to be substrates for both the P-gp and MRP resistance mechanisms (Robson et al., 1998).

VII. Integrated Analysis of Drug Activity: Pharmacoproteomics and Pharmacogenomics

Proteins are the key mediators of cellular behavior, and understanding how proteins direct cellular changes from normal to malignant phenotype and the process of adaptation and resistance to toxicity induced by anticancer agents is of key importance to control these changes and improve treatments. Along with the growing knowledge of genomics, substantial efforts are underway to understand the encoded information, a challenge mainly conceived as *proteomics* (Naaby-Hansen et al., 2001; Whitelegge and Le Coutre, 2001). Proteomics is a newly coined term that refers to the study of the proteome, the protein products of the genome (Wasinger and Corthals, 2002). The opportunities offered by proteomics are not limited to a list of all the proteins; indeed, the scientific target of proteomics is to characterize the flow of information within the cell, which is mediated by networks of proteins that organize in discrete signal transduction pathways (Liotta et al., 2001). The critical issues of anticancer drug sensitivity and toxicity, treatment optimization, and drug target identification are analyzed at the genomic and proteomic levels by *pharmacogenomics* and *pharmacoproteomics*, two distinct disciplines that provide separate and complementary classes of information. Direct analysis of tumor tissues offers obvious advantages since genomic and proteomic analysis of cultured cell lines, when compared with microdissected cells from the same patient, revealed a significant lack of correlation in expression patterns (Liotta et al., 2001). While this was not unexpected, it emphasizes the need for the development of microtechnologies to analyze the proteomic profile in small tumor samples obtained from clinical specimens. Laser capture microdissection provides access to cells directly from tissue specimens; however, the limited availability of patient material urges scientists to develop new highly sensitive methodologies for proteomic profiling of human malignancies. The limitations of two-dimensional gel electrophoresis, the mainstay of most proteomic analysis, are being overcome by new approaches to protein characterization, including high-density protein arrays, antibody arrays, and small molecular arrays (Liotta et al., 2001). Surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry is a novel approach to protein analysis that combines chromatography and mass spectrometry. One of the key features of SELDI-TOF mass spectrometry is its ability to provide a rapid protein expression profile from a variety of biological and clinical samples, and it has been used for biomarker identification and for the study of protein-protein and protein-DNA interaction (Issaq et al., 2002).

The application of protein research to the response of tumors to drugs led to valuable advances in the understanding of pharmacodynamics of anticancer agents. The study of the effect of butyrate, a fatty acid that causes growth arrest and apoptosis of cancer cells, using two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, evidenced that drug treatment resulted in alterations in the proteome of HT-29 cells (Tan et al., 2002). These changes included components of the ubiquitin-proteasome system, and both pro-apoptotic (caspase-4 and cathepsin D) and anti-apoptotic proteins (hsp27, antioxidant protein-2, and pyruvate dehydrogenase E1) were up-regulated in butyrate-treated cells, some of them (cathepsin D and hsp27) showing a time-dependent increase in expression (Tan et al., 2002). Treatment with 5′-azacytidine leads to a decrease in cell growth of lymphoma cell line DG 75 with an arrest at the G_0/G_1 phase of the cell cycle and increased expression of $p16^{INK4a}$ because of promoter demethylation. Proteomic study evidenced that 5′-azacytidine treatment affected protein expression; some of the up-regulated proteins appeared related to the energy metabolism, cytoskeleton organization, cell viability, and protein synthesis (Poirier et al., 2001). The analysis of quantitative changes in the protein composition of the CEM T-lymphoblasts after treatment with a cyclin-dependent kinase inhibitor demonstrated significant down-regulation of α-enolase, triosephosphate isomerase, eukaryotic initiation factor 5A, and α- and β-subunits of Rho GDP-dissociation inhibitor 1. These proteins are known to play an important role in cellular functions such as glycolysis, protein biosynthesis, and cytoskeleton rearrangement (Kovarova et al., 2000).

One additional application of proteome research is the study of the multidrug resistance phenotype in cancer cells, which results from protein-mediated drug detoxification, cellular drug transport, and DNA replication and repair mechanisms (Hutter and Sinha, 2001). Protein analysis has been applied to the study of drug-resistant phenotype toward daunorubicin, mitoxantrone, etoposide, cisplatin, fotemustine, and vindesine (Poland et al., 2002). Finally, proteomic profile analysis appears to be a valuable tool for identification of proteins that may serve as cancer-specific biomarkers for early detection and biologic profiling. Using the approach of laser capture microdissection followed by separation by two-dimensional gel electrophoresis and analysis by mass spectrometry, the 52-kDa FK506 binding protein, Rho G-protein dissociation inhibitor, and glyoxalase I were found to be selectively overexpressed in invasive human ovarian cancer with respect to tumors with low malignant potential (Jones et al., 2002).

VIII. Concluding Remarks

Lung cancer is the most common cause of cancer death worldwide. There is a continued search for novel screening methods and evaluation of molecular events or intermediate biomarkers, not only to detect those subjects at higher risk of developing the disease, but also to assess the likelihood of response to chemotherapeutic treatments and select novel targets for improved therapies (Rosell et al., 2001). In this context, the application of pharmacogenetics and postgenomic techniques has the potential to improve the management of patients with lung cancer, particularly by providing the molecular basis for choosing among the increasing number of chemotherapeutic agents available for the treatment of NSCLC.

Most of the scientific literature available thus far deals with the analysis of few, if not just one, genetic markers of the disease, and this has been the limitation of translational studies on genetics of NSCLC. Genome-wide expression profiling is an important tool for functional genomic studies and represents a unique opportunity to speed up the process of identifying correlations between genotype and clinical course in individual patients. Automated technology allows high-throughput gene activity monitoring by analysis of complex expression patterns, resulting in fingerprints of diseased versus normal or developmentally distinct tissues. Differential gene expression can be most efficiently monitored by DNA hybridization on arrays of oligonucleotides or cDNA clones. Starting from high-density filter membranes, cDNA microarrays have recently been devised in chip format to make gene expression profiling a useful tool for the identification of genetic abnormalities amenable for anticancer drug development (Johnson, 2001). The same cDNA libraries can be used for high-throughput protein expression and antibody screening on high-density filters and microarrays. These libraries connect recombinant proteins to clones identified by DNA hybridization or sequencing, hence creating a direct link between genes and functional proteins. Clone libraries become amenable to database integration including all steps from DNA sequencing to functional assays of gene products (Büssow et al., 2001). In the future, *pharmacoproteomics* will represent a distinct scientific field dedicated to the analysis of the interaction between drugs and protein targets for drug discovery and treatment optimization. This novel technology will allow avoiding the problem of the frequent lack of concordance between gene expression, as evidenced by mRNA levels within the cell, and protein accumulation (Anderson and Seilhamer, 1997). Although most practical proteomics is being done with bidimensional gels and mass spectrometry, including specialized techniques such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for high-throughput screening, there are protein chip technologies under development. One of the first is surface-enhanced laser desorption ionization, which is showing the potential for discovery of novel cancer biomarkers (Vastag, 2000).

Finally, pharmacogenomics, owing to the continuous development of technology and bioinformatics, has the potential to enhance the ability of pharmacologists to increase knowledge of the mechanism of action of drugs and the interaction with the genetic background of individual subjects, and clinicians to use anticancer agents in a safer and more effective manner.

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