

# Oncogenes and Tumor Suppressor Genes in Small Cell Lung Carcinoma

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## 1. Introduction

Small cell lung cancer (SCLC) makes up almost 15% of all cases of lung cancer and occurs almost exclusively in individuals with a history of smoking (Blackhall & Faivre-Finn, 2011; Meyerson et al., 2004; Tamasi and Muller, 2011; Walenkamp et al., 2009). However, SCLCs differ significantly from NSCLCs in specific genetic alterations that occur. Moreover, smoking-damaged bronchial epithelia accompanying SCLCs appears to have undergone significantly more acquired genetic damage than is frequently found in NSCLCs. Two subtypes of SCLC exist: homogeneous small cell carcinoma and combined SCLC (mixture of any non-small cell type) (Meyerson et al., 2004; Tamasi and Muller, 2011). SCLC in its advanced stage has an aggressive clinical course and is commonly accompanied by paraneoplastic syndromes. Autocrine growth factors, such as neuroendocrine regulatory peptides (e.g. bombesin/gastrin-releasing peptide), are prominent in SCLC.

SCLC is categorized as limited stage disease (LS) when confined to the ipsilateral hemithorax and within a single radiation port, while extensive stage disease (ES) includes metastatic disease outside the ipsilateral hemithorax (Blackhall & Faivre-Finn, 2011; Meyerson et al., 2004; Tamasi and Muller, 2011; Walenkamp et al., 2009). SCLC is sensitive to chemotherapy; response rates to front-line agents are often in the range of 60%, with approximately 10% of patients achieving a complete response, even in the setting of metastatic disease (Brambilla et al., 2009; Jemal et al., 2006). Despite this, the relapse rates are quite high and survival with currently available salvage therapy is quite modest. With current therapy, patients with LS-SCLC have a median survival of 17 months and a 5-year overall survival rate of 12% , while patients with ES-SCLC have a median survival of 8.9 months, and a 5-year survival rate of approximately 2%. (Brambilla et al., 2009; Jemal et al., 2006; Tamasi and Muller, 2011). This article will review the molecular targeted agents, the genetic abnormalities, and therapeutic efficacy in SCLC.

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## 2. *Trp53* gene

The *p53* gene located on chromosome 17p13.1 encodes a nuclear protein that acts as a transcription factor and causes cell cycle arrest or apoptosis. Mutations of this gene lead to the loss of tumor suppressor function, thereby promoting cellular proliferation. The majority of the mutations seen in lung cancers are G to T transversions on the non-transcribed strand, suggesting mutagenesis secondary to tobacco smoke. Wild-type p53 protein is present in very low levels in normal cells, whereas mutant p53 is present in much greater quantities in tumor cells due to its prolonged half-life. In fact, 40–70% of SCLC express abnormal p53 protein (Wistuba et al., 2001). Inactivating mutations of *p53*, seen in approximately 90% of SCLC, are typically missense mutations in the DNA binding domain and, to a lesser degree, homozygous deletions (Demirhan et al., 2010). Preclinical studies have shown that a vaccine composed of dendritic cells transduced with a human wild-type *P53* containing recombinant adenovirus (DC-Ad-p53) causes an antitumor response (Ishihda et al., 1999). The differential expression of the mutant *P53* gene between normal and tumor cells could provide a basis for vaccine therapy.

Antonia et al. (2006) treated 29 patients with relapsed/refractory ES-SCLC with a vaccine consisting of dendritic cells transduced with the full-length wild-type *P53* gene delivered via an adenoviral vector. Only one patient showed a clinical response to the vaccine therapy. Interestingly, there was a high rate of objective clinical responses to chemotherapy (61.9%) that immediately followed vaccination. This clinical response to subsequent chemotherapy was closely associated with induction of immunologic response to vaccination. Hence, it is likely that vaccine therapy could serve as an adjunct to chemotherapy, rather than a primary treatment modality. Using this rationale, a current phase I/II trial is evaluating an autologous dendritic cell-adenovirus p53 vaccine following standard platinum-etoposide chemotherapy in patients with ES-SCLC (Horn et al., 2011). Another approach towards vaccination therapy is to target the ganglioside GD3, a cell surface glycosphingolipid antigen that is typically expressed on cells of neuroectodermal origin and a subset of T-lymphocytes (Grant et al., 1999). Studies using SCLC cell lines suggest that these cell lines express significant levels of GD3. The anti-idiotypic antibody BEC-2, which mimics the structure of the GD3 ganglioside, showed promising results in a pilot study (Grant et al., 1999). Based on these results, the European Organization for Research and Treatment of Cancer (EORTC) performed a randomized phase III study in 515 patients with LS-SCLC who were randomized to receive BEC-2 or not as a maintenance treatment following standard induction chemotherapy (Giaccone et al., 2005). Although there was no improvement in overall survival, progression-free survival, or quality of life in the vaccination arm, there was a trend toward prolonged survival in patients who had a humoral response. The authors concluded that vaccination strategies may be warranted with vaccines that were better able to induce a humoral immune response.

Yang et al. (2011) evaluated the role of genetic *P53* polymorphism in radiation-induced pneumonitis (RP), a common dose-limiting toxicity of radiotherapy. In a cohort comprised of 253 (188 NSCLC and 65 SCLC) lung cancer patients receiving thoracic irradiation, the *P53* 72Arg/Arg genotype was associated with increased radiation-induced pneumonitis risk compared with the 72Pro/Pro genotype. Furthermore, the *P53* Arg72Pro and ATM-111G>A polymorphisms displayed an additive effect in intensifying the risk of developing RP. The cross-validation test showed that 63.2% of RP cases could be identified by *P53* and *ATM* genotypes. Thus, genotyping *P53* and *ATM* polymorphisms might help proactively identify

patients susceptible to developing RP when receiving radiotherapy. A recent report by Garcia and co-workers (2010) describe the association of SCLC with ovarian metastases. A 54-year-old woman with SCLC presented with a left ovarian mass, 4.8 cm in diameter, the microscopic appearance of which was identical to the previous bronchoscopic biopsy. Molecular analysis of *P53* demonstrated an identical point mutation (S215) in both tumor sites. Moreover, a *P53* DNA polymorphism (P52R) was identified in normal tissue, but present in homozygosity in both tumor sites.

### 3. Retinoblastoma (*RB*) gene

The *RB* gene, located on chromosome 13q14.11, has been implicated in the regulation of cell cycle progression, particularly the G1 to S-phase transition, in part, through inactivation of members of the E2F transcription factor family (Modi et al., 2000; Schaffer et al., 2010; Wikman et al., 2006). Hypophosphorylated RB is the growth suppressing form that controls the transcription factors E2F1, E2F2 and E2F3, which are necessary for the G1/S transition. (Modi et al., 2000; Wikman et al., 2006). When bound to hypophosphorylated RB, E2F is in its inactive form, causing cell arrest in the G1 phase. The cyclin D1/CDK4 complex phosphorylates RB, which in turn releases E2F, allowing its activation and promoting entry into S phase. During S phase, cyclin E and CDK2 assert control over the phosphorylation of RB (Wikman et al., 2006; Xue et al., 2003). Inactivation of pRB by gross structural alterations or point mutations in the *RB-1* gene has been described in >90% of all SCLC (Wikman et al., 2006; Xue et al., 2003). The types of mutations that occur in the *RB* gene include deletions, nonsense mutations and splicing abnormalities. Phosphorylated RB suppresses apoptosis by repressing other pro-apoptotic target genes, including apoptotic protease activating factor-1 (Apaf-1) and caspases.

There have been numerous reports of RB protein expression in lung cancer (Wikman & Kettunen, 2006). Similar to human tumor entities in general, different lung cancer types show extensively varying expression patterns: SCLC and large cell neuroendocrine carcinomas (LCNEC) are mostly characterized by loss of RB expression (~90%). Conversely, altered expression of RB is rare (~25%) in squamous cell carcinoma (SCC) and adenocarcinoma (AC), which comprise most cases of NSCLC (Leversha et al., 2003; Gouyer et al., 1998). Rather, NSCLC is attributed to *p16<sup>INK4a</sup>* loss or *CCND1* overexpression (Schauer et al., 1994). Interestingly, a few cases of SCLC display *p16<sup>INK4a</sup>* alterations, but retain normal RB. Preinvasive bronchial lesions and carcinoid tumors also rarely exhibit abnormal RB expression. It is thought that SCLC and other neuroendocrine lung tumors originate from the progenitor neural crest that is similar to the origin of RB.

*RB* is altered by mutations (20–30%) or small deletions and chromosomal loss (80–90%) in SCLC (Mori et al., 1990; Kashii et al., 1994). Even though 58% of neuroendocrine lung tumors show low or absent mRNA levels, no hypermethylation has been observed in these tumors. In NSCLC, some reports have detected no *RB* DNA alterations, whereas others, somewhat controversially, have found frequent loss of heterozygosity (LOH) (up to 75%) and mutations (33%) of *RB* in SCC (Gouyer et al., 1998; Leversha et al., 2003). DNA Alterations of *RB* have rarely been described in AC (Sachse et al., 1994). In summary, complete loss of *RB* or a mutant form of *RB* are present in greater than 90% of SCLC cases (Modi et al., 2000). As all normal cells express functional RB, drugs that target cells with inactivated or deleted *RB* would be appropriate candidates for testing in patients with SCLC. Such drugs include heat shock protein-90 (Hsp90) inhibitors (Rodina et al., 2007).

#### 4. *BCL2* gene

*BCL2* is an oncogene that plays a major role in suppressing apoptosis and thus in treatment resistance (Ilievska et al., 2008; Tudor et al., 2000). Therefore, suppression of *BCL2* may increase therapeutic efficacy (Ilievska et al., 2008; Lawson et al., 2010; Ziegler et al., 1997). Since *BCL2* is expressed in the vast majority of SCLC cases, it represents a potential therapeutic target in this disease. G3139 (oblimersen) is an 18-base antisense phosphorothioate oligonucleotide complementary to the *BCL2* mRNA in the region encoding the first six amino acids (Reed et al., 1990). Preclinical and clinical studies have demonstrated that intravenous administration of G3139 reduces *BCL2* protein production (Waters et al., 2000). The combination of oblimersen and paclitaxel was evaluated in a phase II trial of 12 patients with chemo-refractory SCLC (Rudin et al., 2002). There were no objective responses, but four patients had stable disease. The low yield of this small study was attributed to the relatively low doses of both agents; the dose of oblimersen chosen may have been insufficient to suppress expression of the target gene *BCL2*, while paclitaxel had to be given at a dose clearly below that routinely used. Also, since inhibition of *BCL2* expression may increase therapeutic efficacy of cytotoxic agents, this strategy may be more beneficial in patients who responded positively to chemotherapy. In order to test this hypothesis, Rudin et al. (2004) combined oblimersen with etoposide and carboplatin in 16 patients with newly diagnosed extensive stage SCLC. This combination yielded promising results with reasonable toxicity and is currently being studied in a randomized phase III trial to define an exact role for this molecule. Additionally, a small molecule *BCL2* inhibitor, AT101, is currently being evaluated in combination with topotecan in patients with relapsed/refractory SCLC. ABT-263 has also been identified as a Bcl-2 inhibitor in numerous SCLC and leukemia/lymphoma cell lines *in vitro* and *in vivo* (Tahir et al., 2010). In another study, Knoefel et al. (2011) studied the single-nucleotide polymorphism C-938A to assess the potential impact as a genetic marker for response to chemotherapy and outcome prediction in 188 Caucasian SCLC patients. Patients carrying the *BCL2*-938CC genotype showed significantly worse time to progression and overall survival than those with the *BCL2*-938AA genotype. This genetic marker might particularly impact on treatment strategies using *BCL2* antisense approaches.

#### 5. *MYC* genes

*c-MYC*, *N-MYC* and *L-MYC* are proto-oncogenes that code for proteins involved in the regulation of proliferation, differentiation, and apoptosis (Komiya et al., 2011; Paulson et al., 2009; Xion et al., 2011). Amplification of the *c-MYC* oncogene has been observed in various human malignancies (Komiya et al., 2011). Studies in small cell lung cancer have suggested that although amplification of the *MYC* family genes is seen in only about 10% of patients with newly diagnosed SCLC, this proportion increases following treatment (Komiya et al., 2011; Paulson et al., 2009; Xion et al., 2011 ; Barr et al., 1998), suggesting that *MYC* expression increases with resistance of SCLC to therapy. Other studies have suggested that higher levels of expression of the *MYC* gene family may play a significant role in the carcinogenesis of SCLC (Kumimoto et al., 2002). Protein overexpression is seen with *MYC* activation by gene amplification or transcriptional dysregulation (Komiya et al., 2011). In addition, amplification of these genes may have a predictive value, since tumors with *N-MYC* amplification have been associated with poor response to chemotherapy, rapid tumor growth, and short survival (Komiya et al., 2011).

## 6. c-KIT receptor

c-Kit is a tyrosine kinase receptor that, when activated by its ligand stem cell factor (SCF), enhances the growth and survival of hematopoietic cells (Reber et al., 2006; Schneider et al., 2010). Preclinical studies have demonstrated the expression of c-KIT and SCF on almost 70% of SCLC cell lines (Reber et al., 2006), suggesting a possible role in the autocrine/paracrine stimulation of tumor growth. Almost a third of patients with ES-SCLC show evidence of c-kit overexpression by immunohistochemistry (IHC) (Potti et al., 2003; 2005). In a phase II study of imatinib in SCLC, Johnson et al. (2003) enrolled 19 patients with extensive disease who were either treatment-naïve or had a chemo-sensitive relapse. No activity for imatinib was reported, with only one patient showing disease stabilization. A major drawback of this study was that 79% of the enrolled patients lacked c-KIT expression. In an attempt to improve on these findings, the Cancer and Leukemia Group B (CALGB) refined the study design and conducted a similar trial (Dy et al., 2005) in patients with c-KIT overexpression. Despite this, the results were similar to those seen in the previous study, with no observed responses and only one patient with stable disease for 31 weeks. In a third study, imatinib was used as maintenance therapy following cisplatin and irinotecan in patients with ES-SCLC and c-KIT overexpression in tumor tissue (Schneider et al., 2010). Patients who received imatinib did not have any improvement in the progression-free survival, and thus, this strategy did not warrant further investigation. One of the reasons for the inactivity of imatinib in SCLC may be that the putative cells of origin of SCLC are not developmentally dependent on c-KIT (Heinrich et al., 2002), as opposed to hematopoietic stem cells. Another reason could be the absence of activating mutations in patients with SCLC in c-KIT exon 11 that predict for imatinib activity in gastrointestinal stromal tumors (Burger et al., 2003).

## 7. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)

The vascular endothelial growth factor (VEGF) family is comprised of VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGF-E growth factors and their three VEGF receptors (VEGFR 1-3) (Tanno et al., 2004; Wójcik et al., 2010). The VEGF signaling pathway leads to increased proliferation, migration, and invasion of endothelial cells, thus mediating tumor angiogenesis (Tanno et al., 2004; Sattler & Salgia, 2003). VEGF is a key factor in the development of new blood vessels that increases the permeability of microvessels (Thomas et al., 2003). High levels of VEGF have been reported in patients with SCLC, which are associated with tumor stage, disease progression, resistance to chemotherapy and poorer outcomes (Fischer et al., 2007). Studies affecting angiogenesis in SCLC have involved: (1) External inhibitors of angiogenesis, chiefly targeting VEGF and its receptor, (2) Endogenous inhibitors such as interferons, and, (3) Miscellaneous agents, e.g., thalidomide. SCLCs express VEGFR1-3 and VEGFR-2, which are actively involved in tumor growth and invasion (Tanno et al., 2004). The VEGF/VEGFR autocrine signaling pathway mediates proliferation and metastasis, which can be inhibited with the use of monoclonal antibodies against VEGFR-2 and VEGFR-3 in SCLC (Tanno et al., 2004). SU6668, which inhibits VEGFR, c-KIT and FGFR, blocks proliferation and angiogenesis in human lung tumor xenografts (Laird et al., 2000). In a study of 87 patients with SCLC who underwent primary resection followed by adjuvant therapy, microvessel count and expression of VEGF significantly affected survival, thereby establishing a role for angiogenesis in SCLC (Lucchi et al., 2002). Another

preclinical trial demonstrated that ZD6474, a VEGFR-2 and EGFR kinase inhibitor, successfully interfered with VEGF signaling and angiogenesis, leading to decreased proliferation and increased apoptosis in SCLC xenografts. Bevacizumab is a recombinant humanized monoclonal antibody against VEGF that has been approved by the US Food and Drug Administration (FDA) for the treatment of metastatic colon cancer and NSCLC. Preliminary reports of bevacizumab following carboplatin and irinotecan treatment in patients with LS-SCLC have demonstrated response rates, 1- and 2-year survival rates and a safety profile comparable to standard therapy (Raefsky et al., 2005). However, whether the addition of bevacizumab increases progression-free and overall survival is currently unclear and needs to be studied in a randomized phase III trial. VEGF-tyrosine kinase (VEGF-TK) inhibitors have been shown to inhibit downstream signaling pathways that are activated following ligand binding to the VEGF receptor. Multiple agents with VEGF-TK inhibitory activity are being tested for their utility in therapy of various malignancies. A number of small molecules that inhibit VEGFTKs are currently in development, including ZD6474, PTK787/ ZKI222584 (vatalanib), AZD2171, BAY 43-9006 (sorafenib), SU11248 (sunitinib), and AMG706 (Herbst et al., 2005; Morabito et al., 2009). Trials incorporating SU11248 (sunitinib), a multi-targeted TK inhibitor, are being planned in SCLC.

The interferons are a family of naturally-occurring cytokines that have anticancer activity through immunomodulatory and antiangiogenic properties (Blackhall & Shepherd, 2004). Interferons affect endothelial cells by blocking production of basic fibroblast growth factor (bFGF). Patients who achieved a response to chemotherapy were randomized to either interferon maintenance or placebo in different randomized trials. In each of those trials, administration of interferon was associated with considerable toxicity, although two studies showed a trend towards improved survival with interferon treatment (Lebeau et al., 1999). Current trials are investigating the role of interferon either as a vaccine or in combination with cytotoxic chemotherapy in SCLC. Initial reports of maintenance therapy with thalidomide following induction with platinum - etoposide in patients with ES-SCLC have been promising (Conney et al., 2005; Ustuner et al., 2008). This approach needs evaluation in the future.

## 8. Epidermal growth factor receptor (EGFR)

Although EGFR expression has been reported to be low in SCLC, gefitinib, an oral EGFR tyrosine kinase inhibitor, has been shown to inhibit EGFR signaling in SCLC cell lines (Schmid et al., 2010; Tanno et al., 2004). Anecdotal evidence has suggested tumor regression in patients with advanced stage SCLC following treatment with gefitinib (Araki et al., 2005; Okamoto et al., 2006). This suggests a potential role for gefitinib and other agents targeting the EGFR pathway in this disease. Sequist et al. (2011) performed systematic genetic and histological analyses of tumor biopsies from 37 patients with drug-resistant NSCLCs carrying EGFR mutations. All drug-resistant tumors retained their original activating EGFR mutations, and some acquired known mechanisms of resistance including, the EGFR T790M mutation or *MET* gene amplification. Furthermore, some resistant cancers showed unexpected genetic changes, such as EGFR amplification and mutations in the *PIK3CA* gene, whereas others underwent a pronounced epithelial-to-mesenchymal transition. Surprisingly, five resistant tumors (14%) transformed from NSCLC into SCLC, and were sensitive to standard SCLC treatments. Serial biopsies in three patients revealed that genetic mechanisms of resistance were lost in the absence of the continued selective pressure of EGFR inhibitor treatment, and were sensitive to a second round of treatment with EGFR

inhibitors (Sequist et al., 2011). Collectively, these results deepen our understanding of resistance to EGFR inhibitors and underscore the importance of repeatedly assessing cancers throughout the course of the disease.

HER2/neu expression in SCLC has been less well studied. Studies have demonstrated overexpression of HER2/neu by immunohistochemistry in approximately 13–30% of patients with advanced stage SCLC (Micke et al., 2001; Potti et al., 2002, 2003). These studies also found that HER2/neu expression was associated with a poor prognosis for patients with advanced disease. Based on these findings, the anti-HER2/neu monoclonal antibody trastuzumab may be useful as a therapeutic agent in SCLC.

## 9. Matrix metalloproteinase (MMP) inhibitors

Matrix metalloproteinases are involved in extracellular matrix (ECM) degradation (Stetler-Stevenson et al., 1993), a key process in metastasis. Use of protease inhibitors to limit extracellular matrix proteolysis by cancer cells, thereby interfering with tumor cell invasion, would thus be an attractive therapeutic target. In a retrospective analysis, elevated MMP expression was identified as a negative predictor of survival in SCLC (Michael et al., 1999). 60 to 70% of tumor cells stained positive by IHC for MMP-1 and -9, while positive signals for MMP-11, -13, and -14 were observed in 70 to 100% of tumor cells. Expression of MMP-11 and MMP-14 was determined to be an independent negative prognostic factor for Marimastat, a synthetic, orally administered, broad spectrum MMPi with activity against collagenases, gelatinases, and stromolysins (Brown & Giavazzi 1995). The National Cancer Institute of Canada-Clinical Trials Group (NCIC-CTG) and EORTC conducted a randomized placebo-controlled trial of marimastat following induction chemotherapy in 532 patients with sensitive SCLC (Shepherd et al., 2002). The addition of marimastat after induction chemotherapy did not result in improved survival and also had a negative impact on quality of life. These disappointing results may be due to patient selection, as MMP expression was not studied in these patients. The proteinases MMP11 and MMP14 were expressed, a more selective MMPi-targeting these two proteins would arguably be more beneficial. In another study, Jumper et al. (2004) evaluated the relationship between circulating MMP-9 and tissue inhibitor of matrix metalloproteinase (TIMP-1). Thirty one male and female patients with either stage III or IV NSCLC and 17 with either stage III or IV SCLC were compared to 117 age-matched non-smoking controls of both sexes. Prior to any treatment of the patient, a baseline serum sample was obtained from each of the patients for the determination of circulating MMP-9 and TIMP-1. The results indicate that both MMP-9 and TIMP-1 were elevated in the serum of patients with SCLC or NSCLC when compared to the controls. However, the mean values for both MMP-9 and TIMP-1 in the two tumors did not differ. The natural physiological relationship between MMP-9 and the inhibitor TIMP-1 was lost in both SCLC and NSCLC, indicative of abnormal alterations by the tumor. This study suggests that advanced lung cancer alters the normal circulatory pattern of MMP-9 and TIMP-1 a finding that could aid in the understanding of tumor invasion and/or metastasis.

## 10. Sonic hedgehog pathway

The hedgehog (HH) signaling pathway is important during embryonic development and may be involved in development and progression of several human malignancies (Datta &

Datta, 2006; Watkins & Peacock, 2004). *In vitro* studies have demonstrated extensive activation of this pathway in a subset of SCLC cell lines (Watkins et al., 2003). The Hedgehog (Hh) pathway is essential in early lung formation and development through epithelial-mesenchymal interactions (Peacock & Watkins, 2008; Watkins et al., 2003). The signaling cascade is initiated by Hh binding to the Patched-1 receptor (Ptch-1), a twelve transmembrane protein. In the absence of Hh ligand, Ptch-1 constitutively inhibits the seven transmembrane protein Smoothed (Smo), and renders the pathway inactive. However, binding of Hh ligand to Ptch-1 causes the inhibition of Smo to be relieved, which then activates a protein complex and downstream transcription of Hh targets in the nucleus, including Gli-1 and Ptch-1. Present at low levels in the basal layer of bronchial epithelium in the adult, active Hedgehog signaling results in expansion of an intraepithelial cell population during airway regeneration induced by naphthalene injury. In SCLC, there is ligand-dependent activation of the Hedgehog pathway in a juxtacrine fashion, with adjacent cells expressing Sonic hedgehog (Peacock & Watkins, 2008; Watkins et al., 2003). Further, *in vitro* and *in vivo* studies have demonstrated that SCLC can be inhibited by the steroidal alkaloid Hedgehog antagonist, cyclopamine (Watkins et al., 2003; Vestergaard et al., 2006). These data support the presence of a progenitor cell in SCLC that remains chemotherapy-resistant and relies on the Hedgehog developmental pathway, which can be targeted. Gene expression analysis on members of this pathway showed that although the key transcription factor of this pathway, GLI, was only weakly expressed in cell lines, it was expressed on most SCLC tumors studied, thereby suggesting an important role for this pathway for tumor growth *in vivo* (Vestergaard et al., 2006). Thus, the data support the idea that the HH pathway may be an ideal therapeutic target in SCLC. Agents that inhibit this pathway, cyclopamine and its analog KAAD cyclopamine, will soon be in clinical trials.

## 11. mTOR pathway

Mammalian target of rapamycin (mTOR), a downstream mediator in the PI3K/Akt signaling pathway, plays a critical role in the regulation of cell proliferation, survival, mobility and angiogenesis (Schmid et al., 2010). Inhibition of this pathway leads to inhibition of downstream signaling elements, thereby resulting in cell cycle arrest in the G1 phase (Chan 2004). Temsirolimus (CCI-779), an inhibitor of mTOR, is being investigated in a phase II trial in patients with ES-SCLC in remission following platinum-based chemotherapy. Genome-wide gene expression profiling revealed that mutant NRF2 affects diverse molecular pathways including the mTOR pathway. Mutant NRF2 upregulates RagD, a small G-protein activator of the mTOR pathway, which was also overexpressed in primary lung cancer (Shibata et al., 2010). Preliminary results show a prolongation of progression-free survival, thereby suggesting significant activity for this agent in SCLC (Pandya et al., 2005). Another agent from this class, everolimus (RAD-001), is currently being tested in a phase II trial for patients with relapsed SCLC (Schmid et al., 2010).

## 12. CD56 (NCAM)

The neural cell adhesion molecule (NCAM, CD56) is associated with the immunoglobulin family and modulates neuroendocrine cell growth, migration, and differentiation (Jensen & Berthold, 2007; Kim and Kwon, 2010). CD56 is an isoform encoded by the NCAM gene. NCAM is found in almost 100% of SCLC and is also expressed on natural killer cells,



neuroendocrine glands, cardiomyocytes, and in the central and peripheral nervous system (Kim and Kwon, 2010). Since malignant cells can be influenced by NCAM signaling, it has been investigated as a target for anti-cancer therapy (Jensen & Berthold, 2007). N901 is an anti-CD56 monoclonal antibody covalently linked to a blocked ricin molecule binds to SCLC tumors and cell lines (Lynch, 1993). Initial studies showed promising activity, but were hampered by the immune response that developed against the murine monoclonal antibody and the ricin molecule, leading to potentially fatal side effects (Fidias et al., 2002). In order to overcome this, a humanized version of this antibody covalently bound to the maytasinoid (microtubule- depolymerizing compound) effector molecule DM-1 has now been made available (BB-10901 or huN901-DM1). Initial studies of this compound have shown evidence of efficacy and safety (Fossella et al., 2005) and a phase II trial is currently underway.

### 13. Chromosomal alterations

In SCLC and other epithelial tumors, multiple chromosomal aberrations are found, reflecting genomic instability (Balsara & Testa, 2002; Sato et al., 2007). Loss of the short arm of chromosome 3 has been consistently seen in SCLC (Balsara & Testa, 2002). The deletion of 3p leads to inactivation of three putative tumor-suppressor genes. The majority of SCLCs have deletions affecting multiple chromosomal sites, with recurrent losses at 3p, 5q, 13q and 17p, which are loci with tumor suppressor genes including p53 (Balsara & Testa, 2002). Comparative genomic hybridization analyses have revealed that a large number of SCLCs harbor gains of 1p, 2p, 3q, 5p, 8q and 19p, regions which encode well-known oncogenes, such as *MYC* and *KRAS*. SCLC cell lines found to have amplifications of 1p, 2p and 3q, and deletion of 18q display a more aggressive phenotype of the disease (Balsara & Testa, 2002). Allele loss on chromosome 3p occurs with a frequency greater than 90% in SCLC and is believed to be an early event found in lung carcinogenesis (Sato et al., 2007). The loss of the fragile histidine triad (*FHIT*) gene results in the accumulation of diadenosine tetraphosphate, stimulating DNA synthesis and proliferation (Sozzi et al., 1996). This gene has been localized to 3p14.2 and is believed to be an important tumor suppressor gene involved in the pathogenesis of lung cancer (Sozzi et al., 1996; Wistuba et al., 2001). The second tumor suppressor gene is believed to be *RASSF1A*, which is located within a 120 kilobase region of chromosome 3p21.3 which also contains the *FUS1*, *SEMA3B* and *SEMA3F* loci. *RASSF1A* encodes a protein similar to RAS effector proteins and is inactivated by tumor-acquired promoter hypermethylation in 90-100% of SCLC samples (Burbee et al., 2001; Dammann et al., 2000). *ASSF1* is involved in cell cycle pathways, apoptosis and microtubule stability (Agathangelou et al., 2005).

*FUS1* is a novel tumor suppressor gene identified in the human chromosome 3p21.3 region where allele losses and genetic alterations occur early and frequently for many human cancers. Expression of *FUS1* protein is absent or reduced in the majority of lung cancers and premalignant lung lesions. Specifically, expression of the *FUS1* protein is lost in 100% of SCLC cases. Interestingly, restoration of wild-type *FUS1* function in 3p21.3-deficient non-small cell lung carcinoma cells significantly inhibited tumor cell growth by induction of apoptosis and alteration of cell cycle kinetics (Ji & Ross, 2008). They report that *FUS1* induces apoptosis through the activation of the intrinsic mitochondrial-dependent and Apaf-1-associated pathways and inhibits the function of protein tyrosine kinases including EGFR, PDGFR, AKT, c-Abl, and c-Kit. Moreover, intravenous administration of a nanoparticle encapsulated *FUS1* expression plasmid effectively delivered *FUS1* to distant

tumor sites and mediated an antitumor effect in orthotopic human lung cancer xenograft models (Ji & Ross, 2008). This approach is the rationale for an ongoing FUS1-nanoparticle-mediated gene delivery clinical trial for the treatment of lung cancer.

Deletion of a third gene, *TGFBR2*, located at 3p21.3.22, that encodes the transforming growth factor  $\beta$  (TGF- $\beta$ ) type II receptor, has also been described in SCLC (Hougaard et al., 1999). This nonsense mutation results in the synthesis of a truncated receptor and has been linked to exposure to benzo[a]-pyrene, a component of cigarette smoke. The gene coding for Retinoic Acid Receptor Beta (*RAR- $\beta$* ) is located on chromosome 3p24 (Mattei et al., 1991). Retinoic acid plays an important role in lung development and differentiation, acting primarily via nuclear receptors. Loss of heterozygosity of *RAR-B2* and *RAR-B4* isoforms is seen in almost all cases of SCLC. Methylation of the promoter region of these two isoforms may be responsible for the silencing of their expression in SCLC (Virmani et al., 2000).

#### 14. Telomerase

Telomeres are genetic elements present at the ends of linear chromosomes that play an important role in stabilizing chromosomes from degradation and cell death (Counter et al., 1992; Hyer & Silvestri, 2000). Telomerase is a ribonucleoenzyme that compensates for telomere shortening during cell division by synthesizing hexameric TTAGGG repeats at the end of the chromosomes. The functional unit of this enzyme consists of an RNA component hTR, and a catalytic component hTERT. Repression of telomerase in the somatic tissues of humans seems to have evolved as a powerful protective barrier against carcinogenesis (Newbold, 2002). Studies in patients with neuroendocrine lung tumors have demonstrated upregulation of the RNA component of telomerase in 98% of human SCLC (Sarvesvaran et al., 1999). Similar studies assessing telomerase activity in small cell lung cancer showed increased activity in all specimens analyzed (Hiyama et al., 1995). Zaffaroni et al. (2003) studied telomerase activity by the telomeric repeat amplification protocol (TRAP) assay in 38 neuroendocrine (NE) lung tumors. A positive TRAP signal was observed in 14 of 15 (93%) SCLCs, 7 of 8 (87%) large-cell NE carcinomas, and only 1 of 15 (7%) typical carcinoid tumors. When telomerase activity was correlated with the gene product-based immunophenotypic profile of individual tumors, the absence of telomerase activity was associated with a lack of BCL-2, P53, c-KIT, and CDK4 expression and presence of RB. Such a phenotype was appreciable in most of the carcinoid tumors. Conversely, telomerase-positive tumors generally showed an immunophenotype consistent with gene product alterations (including high expression of BCL-2, P53, and c-KIT, and loss of RB) and were characterized by a high proliferative index. These data support the previously reported evidence for two genetically unrelated groups of NE lung tumors that have distinct phenotypic profiles (Zaffaroni et al., 2003).

#### 15. Knockout Mouse Models for SCLC

Contrary to NSCLC, neuroendocrine carcinomas are virtually never found in spontaneous or chemically induced murine lung cancer. One reason for this could be that in these murine models, a combination of both *p53* and *Rb* mutations is almost never found, unlike most human SCLCs. To address this issue, a Cre/lox based deletion of both conditional alleles for *Rb* and *p53* was performed by intratracheal instillation with Adeno-Cre (Meuwissen et al., 2003). After 3 months, foci of neuroendocrine hyperplasia developed through the proximal

as well as distal bronchi. After a further 3 months, these early lesions progressed into massive lung tumors with histological features typical of SCLC. Interestingly, some early type lesions remained even in the presence of extensive SCLC. Consequently, it was of importance to determine if the early neuroendocrine lesions are indeed precursors for SCLC and, if so, which additional epigenetic events are then needed for progression. Immunohistological characterization of the full-blown tumors revealed that they indeed shared neuroendocrine features with human SCLC. As in human SCLC all neuroendocrine differentiation markers, such as calcitonin gene-related protein (CGRP), neuron-specific enolase, synaptophysin, neural cell adhesion molecule, and achaete-scute homolog-1 (ASH-1) were not expressed. Furthermore, the murine SCLC readily metastasized towards similar organs as found with human SCLC (Meuwissen et al., 2003). All primary SCLC, as well as their metastases, had all *Rb* and *p53* alleles inactivated. Tumors that retained one WT *Rb* allele were all invariably adenocarcinomas without any neuroendocrine features. Therefore, the status of *Rb* most likely determines if tumors occur with mixed SCLC and NSCLC phenotypes, as has been observed in some patients (Brambilla et al., 2000). No lung tumors were found in *Rb<sup>F/F</sup>* mice, which suggested that loss of *Rb* alone is not enough to initiate lung tumorigenesis (Meuwissen et al., 2003) and the additional loss of *p53* is needed.

Not only does *Rb* loss require additional genetic events to initiate lung tumorigenesis, the nature of these complementary lesions also determines which type of lung cancer will develop. For instance, *RB* inactivation and *KRas* mutations are almost never found together in the same human lung cancer. Moreover, the overall mutation rate of *RB* in human NSCLC is very low (Wistuba & Gazdar, 2003). As noted, Adeno-Cre dependent activation of *Kras* in a broad range of lung epithelial cells leads exclusively to the onset of NSCLC. However, when *LSLKrasG12D* transgenes were combined with *Rb<sup>F/F</sup>* and *Rb* family *p130<sup>F/F</sup>* alleles for Adeno-Cre-dependent lung tumor induction, much more advanced adenocarcinomas of *Kras<sup>G12D</sup>; Rb<sup>-/-</sup>; p130<sup>-/-</sup>* genotypes resulted compared to single *Kras<sup>G12D</sup>* (Ho et al., 2009). Loss of both *Rb* and *p130*, albeit to a lesser extent, contributed to *Kras* dependent NSCLC. Clearly, in this genetic context *Kras<sup>G12D</sup>* overrules any effect of *Rb* loss on neuroendocrine differentiation.

Another intriguing observation came from *CC10-rtTA; tetO7-Cre; Rb<sup>F/F</sup>* mice administered doxycycline during early embryogenesis, causing a complete *Rb* ablation in all bronchial Clara cells. However, only increased hypercellular neuroendocrine lesions were detected in these mice, and no effect on Clara cell homeostasis could be observed. Alternatively, when all three *Rb* family proteins (*Rb*, *p107* and *p130*) were inactivated by a truncated SV40 large T-antigen oncoprotein (T121) in *CC10-T121* mice, severe bronchial hyperplasia with complete dedifferentiation of all Clara cells occurred. These results suggest that *Rb* might be specifically required for determining neuroendocrine cell fate, but only in a strict cellular and genetic context. The combined evidence from mouse models indicate it is unlikely that NSCLC and SCLC develop from similar target cells. It would be more plausible that separate, non-identical target cells can develop into different lung cancers, although each still depends on specific major genetic pathways.

Apart from the somatic *Rb<sup>F/F</sup>; p53<sup>F/F</sup>* model for SCLC, two other lung cancer models have also been associated with pulmonary neuroendocrine tumors. One model made use of bitransgenic *CC10-hASH1; CC10-SV40 large T* system in which progressive neuroendocrine dysplasia and aggressive lung adenocarcinoma developed with both focal neuroendocrine differentiation (through expression of pro-neural ASH-1 transcription factor) and *CC10* expression (Linnoila et al., 2000). These adenocarcinomas closely resembled human NSCLC

with neuroendocrine differentiation (Linnoila et al., 1994). In the other model, the cyclin dependent kinase inhibitor *p18<sup>Ink4c</sup>* and *Men1*, a tumor suppressor gene deleted in human multiple endocrine neoplasia, were both inactivated. To determine how *p18* and *p27* genetically interact with *Men1*, Pei et al. (2007) characterized *p18-Men1* and *p27-Men1* double mutant mice and showed that *p18*, but not *p27*, functionally collaborates with *Men1* in suppressing lung tumorigenesis. Lung tumors developed in both *Men1<sup>+/-</sup>* and *p18<sup>-/-</sup>*; *Men1<sup>+/-</sup>* mice at a high penetrance and contained both neuroendocrine and non-neuroendocrine cells. The remaining wild-type *Men1* allele was lost in most lung tumors from *Men1<sup>+/-</sup>* mice, but was retained in most tumors from *p18<sup>-/-</sup>*; *Men1<sup>+/-</sup>* mice, showing a functional collaboration between *p18* and *Men1* in lung tumor suppression (Pei et al., 2007). Phosphorylation of Rb protein at both Cdk2 and Cdk4/Cdk6 sites were significantly increased in normal bronchial epithelia and tumor cells derived from *p18<sup>-/-</sup>*; *Men1<sup>+/-</sup>* mice compared to those from single *p18<sup>-/-</sup>* or *Men1<sup>+/-</sup>* mice. Lung tumors developed in *p18<sup>-/-</sup>*; *Men1<sup>+/-</sup>* mice were multifocal, more heterogeneous, and highly invasive compared to those in either *p18<sup>-/-</sup>* or *Men1<sup>+/-</sup>* mice. These results revealed a previously unrecognized function of *p18* in lung tumor suppression through collaboration with *Men1* to control lung stem cell proliferation. To investigate the cellular origin(s) of this cancer, Sutherland et al. (2011) assessed the effect of *Trp53* and *Rb1* inactivation in distinct cell types in the adult lung using adenoviral vectors that target Cre recombinase to Clara, neuroendocrine (NE), and alveolar type 2 (SPC-expressing) cells. Using these cell type-restricted Adeno-Cre viruses, loss of *Trp53* and *Rb1* efficiently transformed NE and SPC-expressing cells, leading to SCLC, albeit SPC-expressing cells were transformed less efficiently (Sutherland et al., 2011). In contrast, Clara cells were largely resistant to transformation. Their results indicate that although NE cells serve as the predominant cell of origin of SCLC, a subset of SPC-expressing cells are also endowed with this ability.

Increasingly, it is realized that during tumorigenesis a variety of cells are recruited into the tumor to provide a range of functions that are associated with tumor progression (Calbo et al., 2011). Karnoub and colleagues (2007) showed that mesenchymal stem cells recruited into the stroma of breast cancer promote metastasis through CCL5-mediated paracrine signaling, thereby emphasizing the relevance of the interactions between tumor cells and the surrounding microenvironment. Using the mouse model for simultaneous *Rb* and *p53* inactivation, Calbo et al. (2011) established primary cultures from 21 murine NSCLCs. They found mouse SCLC primary cultures attached to the dishes in 9 of 21 cases, consistent with cell culture derived from human SCLC. They seeded single-cell suspensions from 15 mouse SCLC cultures in soft agar-containing medium, and isolated individual colonies and expanded separately. Most of the obtained clones grew as suspending aggregates of very small cells. These cells expressed neuroendocrine protein markers such as synaptophysin, achaete-scute complex homolog 1, and neural cell adhesion molecule. They also obtained clones grew as a cellular monolayer composed of larger cells with visible cytoplasm and spindle-like membrane extensions, spreading on the substrate (mesenchymal cells). This is consistent with human SCLC that are often composed of phenotypically different cells with either a neuroendocrine or a mesenchymal marker profile. Importantly, these two types of cells had a common origin because they shared specific genomic aberrations as demonstrated by SKY analysis. Calbo et al. also showed that the transition from neuroendocrine to mesenchymal phenotype could be achieved by the ectopic expression of oncogenic Ras<sup>V12</sup> in the former (Calbo et al., 2011). When engrafted as a mixed population, the mesenchymal cells endowed the neuroendocrine cells with metastatic capacity,

illustrating the potential relevance of tumor cell heterogeneity in dictating tumor properties (Calbo et al., 2011). In short, they showed a specific type of tumor heterogeneity of SCLC, in which the interaction between clonally derived but diversified subclones alters the behavior of the tumor as a whole (Calbo et al., 2011). One outcome was a substantially increased metastatic potential, a feature with important clinical ramifications in human SCLC. Tumor cell behavior then not only depended on the interactions with stromal cells, but was also influenced by interactions with tumor cell variants that fulfill a distinct role in the tumor tissue. This study provides a unique system, whereby the mesenchymal compartment of the tumor was generated from a separate subclone of SCLC during the tumorigenic process, providing the tumor cell population as a whole with new capabilities such as metastatic potential.

## 16. Xenograft models for SCLC

For the past three decades, the mainstay of preclinical cancer therapeutic research has been the use of human cancer cells lines cultured *in vitro* and of xenografts derived from these cell lines grown *in vivo* in immunodeficient mice. However, neither model consistently predicted the efficacy in clinical trials, resulting in two major barriers to the successful translation of new cancer therapeutics. First, resources are expended on drug development based on these models that ultimately fail in clinical trials. Second, many potentially useful therapies that might be beneficial in humans are discarded because the animal models fail to demonstrate efficacy in conventional cell culture and xenograft models. Emerging evidence suggests that the process of establishing conventional cell lines from human cancers results in distinct and irreversible loss of important biological properties. These include (a) gain or loss of gene amplification, (b) the ability to migrate and metastasize, (c) the maintenance of a distinct stem cell population, and (d) the preservation of dependency on embryonic signaling pathways. These properties are not restored when these conventional cell lines are grown as heterotopic or orthotopic xenografts.

Because SCLC is usually diagnosed by endobronchial biopsy or fine-needle aspiration cytology, substantial quantities of fresh or frozen tissues are typically lacking in most tumor banks. For this reason, most SCLC researches rely on conventional cell lines, which are often chemoresistant because they were derived from patients who had received cytotoxic chemotherapy (Phelps et al., 1996). In addition, all of these cell lines have experimental limitations and lack the three-dimensional tumor-stromal interactions, which seem to significantly affect the response of these cells to chemotherapy (Hodkinson et al., 2007). To establish better models for the study of SCLC, Daniel et al. (2009) generated and characterized a series of primary xenograft models derived from chemo-naïve patients to more accurately model SCLC in mice. In parallel, cell lines grown in conventional tissue culture conditions were derived from each xenograft line, passaged for 6 months, and then reimplanted to generate secondary xenografts. When compared with normal lung, primary tumors, xenografts, and cell lines displayed a gene expression signature specific for SCLC (Daniel et al., 2009). Comparison of gene expression within the xenograft model identified a group of tumor-specific genes expressed in primary SCLC and xenografts that was lost during the transition to SCLC cell lines; these genes were not regained when the tumors were re-established as secondary xenografts. Such changes in gene expression may be a common feature of many cancer cell culture systems, with functional implications for the use of such models for preclinical drug development.

Bcl-2 is a central regulator of cell survival that is overexpressed in most SCLC tumors and contributes to both malignant transformation and therapeutic resistance. Hann et al. (2008) compared primary SCLC xenografts prepared from *de novo* human tumors with standard cell line-based xenografts to evaluate a novel and highly potent small molecule inhibitor of Bcl-2, ABT-737. ABT-737 induced dramatic regressions in tumors derived from some SCLC cell lines. In contrast, only one of three primary xenograft SCLC tumors showed significant growth inhibition with ABT-737. Explanations for this apparent difference may include relatively low expression of Bcl-2 in the primary xenografts or inherent differences in the model systems. The addition of etoposide to ABT-737 in the primary xenografts resulted in significantly decreased tumor growth, underscoring the clinical potential of ABT-737 in combination therapy. To identify factors that may contribute to resistance to ABT-737 and related inhibitors, they isolated resistant derivatives of an initially sensitive cell line-based xenograft. Acquired resistance in this model was associated with decreases in the expression of the primary target Bcl-2, of proapoptotic partners of Bcl-2 (Bax and Bim), and of Bcl-2:Bim heterodimers. Expression profiling revealed 85 candidate genes demonstrating consistent changes in gene expression with acquired resistance. These data have specific implications for the clinical development of Bcl-2 inhibitors for SCLC and broader implications for the testing of novel anticancer strategies in relevant preclinical models.

## 17. Conclusions

There are many distinct genetic pathways present in SCLC, leading to its unique biology and clinical features. A better understanding of these basic molecular and cellular changes will allow for the development of novel therapeutic strategies. Multiple molecularly targeted agents are actively being studied pre-clinically and clinically, with the hope of ultimately improving survival of patients with SCLC. Development of targeted therapy in small cell lung cancer has significantly lagged behind that of non-small cell lung cancer. Etoposide and cisplatin remain the mainstays of first-line SCLC treatment. Although the decreasing prevalence of smoking in industrialized countries will lead to decreased incidence of SCLC, the burden of disease is shifting to developing countries. Further investment in research for this disease is, therefore, warranted. Many phase 1 and 2 studies of drugs with potential activity in SCLC and phase 2 and 3 trials to improve radiotherapy are underway. Inclusion of patients with SCLC in such trials should be encouraged, especially otherwise healthy patients with relapsing or refractory SCLC, for whom treatment options are limited. A new, effective, and active combination for extensive-stage SCLC would be quickly moved up as a treatment priority.

SCLC remains a therapeutic challenge despite high initial responses to chemotherapy and radiotherapy. The fact that several promising molecularly targeted agents have not shown adequate activity in clinical trials does not mean the end of novel targeted therapies for SCLC. Nevertheless, a better understanding of SCLC biology and better preclinical models of SCLC are needed to improve available therapies.

The mouse model presented in the study by Meuwissen et al. clearly showed that somatic inactivation of both *Rb1* and *Trp53* alleles in lung epithelial cells readily leads to formation of small cell NE tumors. Their histopathologic characteristics and metastasizing capacity were strikingly similar to human SCLCs. Their mouse model will prove a valuable tool for (1) comparing genotype-phenotype similarities between human SCLC and MSCLC, (2) the identification of precursor lesions, and (3) additional factors involved in tumor progression,

and (4) ultimately, testing of targeted, novel tumor intervention strategies and chemoprevention.

The identification of specific cell type(s) from which SCLC originates is critical in the development of methods for early diagnosis and treatment. By using cell type-restricted Adeno-Cre vectors in directing *Trp53* and *Rb1* loss to distinct cell populations in the adult mouse lung, Sutherland et al. showed that NE cells are the predominant cells of origin of SCLC. Their study provides additional tools to address questions related to the cell of origin of lung cancer, and highlights the importance of specifically targeting NE cells for the treatment of SCLC. Their strategy to manipulate specific adult lung cell populations in a controlled manner by cell type-restricted somatic gene transfer vectors could help to answer the question of whether distinct lung pathologies have a unique cell of origin, and whether this cell of origin is a determining factor in the drug resistance profile of the various tumor subtypes.

The work of Calbo et al. showed a specific type of tumor heterogeneity, in which the interaction between clonally derived but diversified subclones alters the behavior of the tumor as a whole. One outcome was substantially increased metastasis, a feature with important clinical ramifications. Thus, tumor cell behavior not only depends on the interactions with stromal cells, but also interactions with tumor cell variants that fulfill a distinct role in the tumor tissue. Enhanced metastatic capacity serves as an illustrative example of crosstalk between specialized tumor cell clones. Disrupting the paracrine signaling involved in this interaction is worth further exploring as a strategy to mitigate tumor progression in SCLC.

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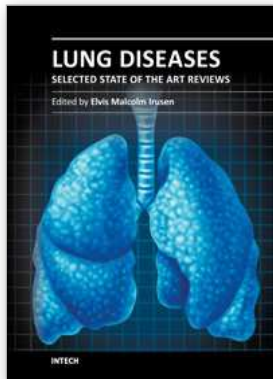
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The developments in molecular medicine are transforming respiratory medicine. Leading clinicians and scientists in the world have brought their knowledge and experience in their contributions to this book. Clinicians and researchers will learn about the most recent advances in a variety of lung diseases that will better enable them to understand respiratory disorders. This treatise presents state of the art essays on airways disease, neoplastic diseases, and pediatric respiratory conditions. Additionally, aspects of immune regulation, respiratory infections, acute lung injury/ARDS, pulmonary edema, functional evaluation in respiratory disorders, and a variety of other conditions are also discussed. The book will be invaluable to clinicians who keep up with the current concepts, improve their diagnostic skills, and understand potential new therapeutic applications in lung diseases, while scientists can contemplate a plethora of new research avenues for exploration.

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