LONG-TERM SMOKING-MEDIATED DOWNREGULATION OF SMAD3 INDUCES TUMORIGENICITY AND CARBOPLATIN RESISTANCE IN NON-SMALL CELL LUNG CANCER

By

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DEDICATION

To my father,

Jay Narayan Samanta

and mother,

Mita Samanta

and other family members

who gave me

unconditional love and support

and had so much faith in me.

Thank you.

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Chapter

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LIST OF ABBREVIATIONS

A	**Adenine**
AhR	**Arylhyrdrogen Receptor**
ATCC	**American Tissue Culture Collection**
B[a]P	**Benzopyrene**
C	**Cytosine**
CSC	**Cigarette Smoke Condensate**
CO_2	**Carbon dioxide**
DMSO	**Dimethylsulfoxide**
DNA	**Deoxyribo-nucleic acid**
EGFR	**Epidermal Growth Factor Receptor**
ELISA	**Enzyme-linked immunoassay**
ERK	**Extracellular-signal regulated kinase**
FBS	**Fetal Bovine Serum**
G	**Guanine**
HDAC	**Histone Deacetylase**
IC_{50}	**Inhibition Concentration by 50%**
q-RT-PCR	**Quantitative real time polymerase chain reaction**
MAPK	**Mitogen-activated protein kinase**
mRNA	**messenger RNA**
MTT	**3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide**

NSCLC	**Non small-cell lung cancer**
PBS	**Phosphate Buffered Saline**
PCR	**Polymerase chain reaction**
PI	**Propidium Iodide**
RNA	**Ribonucleic acid**
RPMI	**Roswell Park Memorial Institute**
siRNA	**Small interfering RNA**
SLC	**Small-cell lung cancer**
SNP	**Single Nucleotide Polymorphism**
T	**Thymine**
TGF-β	**Transforming growth factor β**
TMA	**Tissue microarray**

CHAPTER I

INTRODUCTION

Lung Cancer - the statistics

Global cancer statistics indicate that lung cancer is responsible for over 1 million deaths each year (Siegel, Naishadham et al. 2012). According to the cancer statistics in US, it is estimated that 221,130 new patients were diagnosed with lung cancer in 2011, accounting for about 14% of cancer diagnoses. The incidence rate is declining significantly in men, from a high of 102.1 cases per 100,000 in 1984 to 71.8 cases in 2007. In women, the rate has begun to decrease after a long period of increase. Even though the incidence of lung cancer is second to prostrate in case of males and breast in case of females the trend of death is different. Lung cancer accounts for more deaths than any other cancer in both men and women. An estimated 156,940 deaths, accounting for about 27% of all cancer deaths, were expected to occur in 2011. For more than a decade since 1987, more women have died each year from lung cancer than from breast cancer. From Figure1 it is clear that the decrease in death rates began in men in 1991. The decrease in death rates in men accelerated to 3.0% per year in 2005. Female lung cancer death rates have been decreasing by 0.9% per year since 2003 after continuously increasing since at least 1930. The difference between the incidence rates and the death rates, with regard to lung cancer, clearly designates the improvements we need to make regarding the treatment of lung cancer by knowing more about lung cancer biology. Gender differences in lung cancer mortality patterns reflect historical differences in

uptake and reduction of cigarette smoking between men and women over the past 50

years (Siegel, Naishadham et al. 2012).
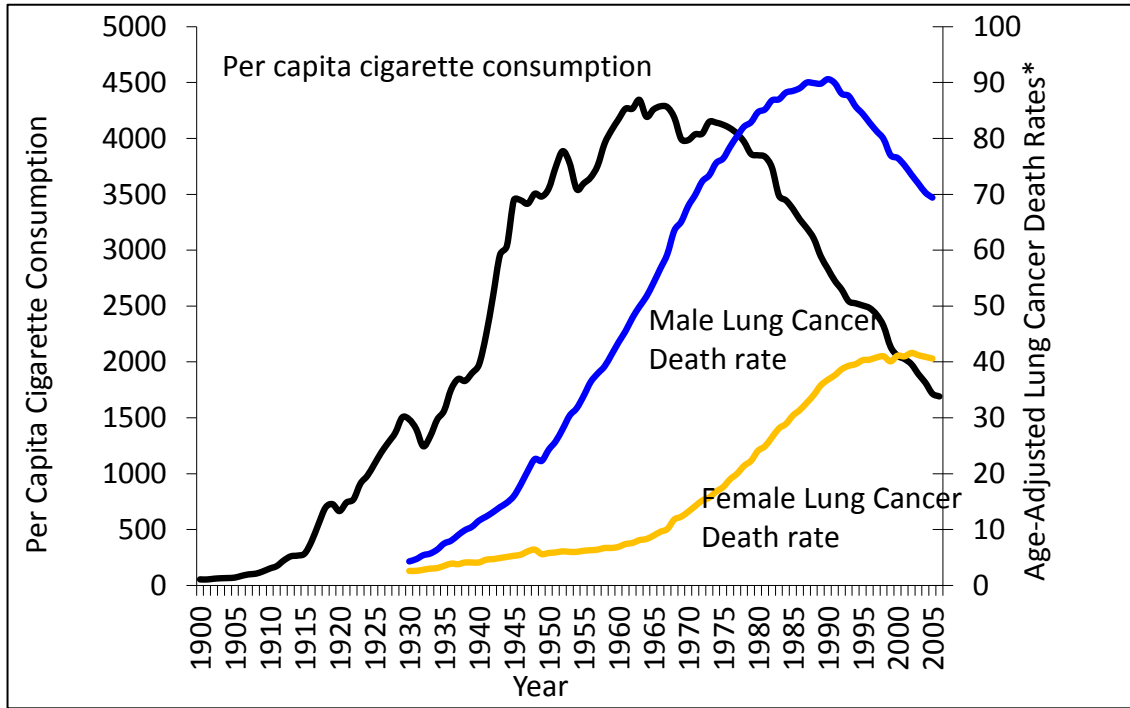
Figure 1. Trend of cigarette consumption and lung cancer deaths.

As a result of the cigarette smoking epidemic, lung cancer death rates showed a steady

increase through 1990, then began to decline among men. The lung cancer death rate

among US women, who began regular cigarette smoking later than men, has begun to

plateau after increasing for many decades. Source: Death rates: US Mortality Data, 1960-

2005, US Mortality Volumes, 1930-1959, National Center for Health Statistics, Centers

for Disease Control and Prevention, 2006. Cigarette consumption: US Department of

Agriculture, 1900-2007. Figure originally published in (Jemal, Bray et al. 2011).

Histological Classification and progression

For the purposes of treatment there are two major forms of lung cancer are non–small-cell lung cancer (NSCLC) (about 85% of all lung cancers) and small-cell lung cancer (SLC) (about 15%). Non–small-cell lung cancer can be divided into three major histologic subtypes: squamous-cell carcinoma, adenocarcinoma, and large-cell lung cancer. Smoking causes all types of lung cancer but is most strongly linked with small-cell lung cancer and squamous-cell carcinoma; adenocarcinoma is the most common type in patients who have never smoked (Herbst, Heymach et al. 2008). Figure 2 shows the linear progression of squamous-cell carcinoma from normal epithelium to hyperplasia, squamous metaplasia, dysplasia, carcinoma in situ and finally to invasive carcinoma (Wistuba and Gazdar 2006). Similar to the adenocarcinoma sequence of colorectal cancer, lung adenocarcinoma is thought to follow a linear progression in which a precursor lesion progresses to adenocarcinoma in situ (Yatabe, Borczuk et al. 2011) as shown in Figure 3.
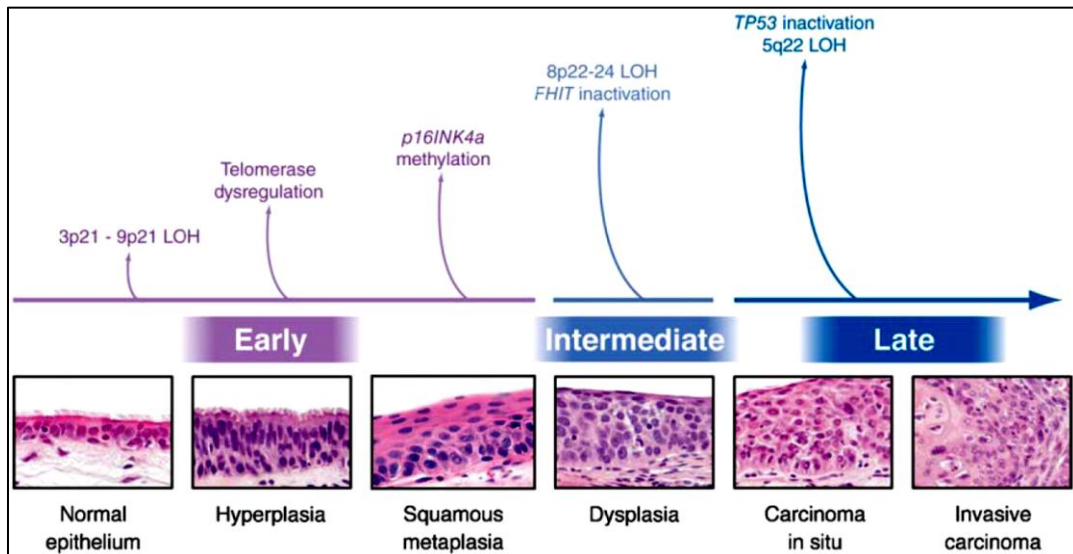
Figure 2. Step wise histopathological and molecular changes during the pathogenesis of squamous cell carcinoma of the lung. Figure originally published in (Wistuba and Gazdar 2006).

Novel non-Linear Progression Schema
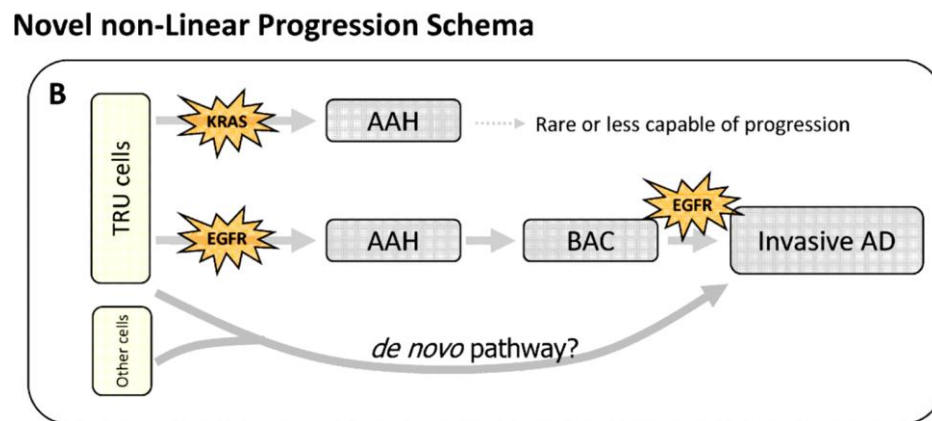
Figure 3. Progression models of adenocarcinoma:

The figure shows a comparison between current linear progression schema and novel non-linear progression scheme of lung adenocarcinoma. Current model follows a single linear progression regardless of the genes involved, whereas the novel model has several pathways to invasive adenocarcinoma, some of which may be terminated prior to invasive adenocarcinoma. Figure originally published in (Yatabe, Borczuk et al. 2011).

Genetic Risk Factors

Not all cigarette smokers develop lung cancers. Currently, there is an attempt to identify the genetic factors that may contribute to the development of lung cancer. Lung-cancer susceptibility and risk are also increased in inherited cancer syndromes caused by rare germ-line mutations in p53 (Hwang, Cheng et al. 2003), retinoblastoma (Sanders, Jay et al. 1989) , and other genes (Bailey-Wilson, Amos et al. 2004) as well as a germ-line mutation in the epidermal growth factor receptor (EGFR) gene (Bell, Gore et al. 2005). More recently, three large genome-wide association studies identified an association between single-nucleotide polymorphism (SNP) variation of the long arm of the chromosome 15q24/15q25.1 and susceptibility to lung cancer. The region of the SNP variation was recently linked to lung carcinogenesis and includes two genes encoding subunits of the nicotinic acetylcholine receptor alpha, which is regulated by nicotine exposure (Lam, Girard et al. 2007; Amos, Wu et al. 2008; Hung, McKay et al. 2008; Thorgeirsson, Geller et al. 2008). The three large genome-wide studies are appropriately replicated and provide a strong evidence for an association between SNP variation at 15q24/15q25.1 and lung cancer. But they differ whether the connection is direct or mediated via smoking behavior.

Lung-cancer susceptibility and risk also increase with reduced DNA repair capacity or increased expression of DNA synthesis and repair genes (Spitz, Wei et al. 2003). Reduced DNA repair capacity from germ-line alterations in nucleotide excision repair genes such as ERCC1 in non-small cell lung cancer correlates with better prognosis (Yu, Zhang et al. 2008). Similarly, increased expression of DNA synthesis and repair genes, including RRM1 (the regulatory subunit of ribonucleotide reductase) in

non– small-cell lung cancer correlates with a better overall prognosis (Zheng, Chen et al. 2007). Figure 4 below presents gene abnormalities involved in the development of different histologic types of lung cancer.

Abnormality		Non–Small-Cell Lung Cancer	Small-Cell Lung Cancer
	Squamous-Cell Carcinoma	Adenocarcinoma	
Precursor			
Lesion	Known (dysplasia)	Probable (atypical adenomatous hyperplasia)	Possible (neuroendocrine field)†
Genetic change	*p53* mutation	*KRAS* mutation (atypical adenomatous hyperplasia in smokers), *EGFR* kinase domain mutation (in nonsmokers)	Overexpression of c-MET
Cancer			
KRAS mutation	Very rare	10 to 30%‡	Very rare
BRAF mutation	3%	2%	Very rare
EGFR			
Kinase domain mutation	Very rare	10 to 40%‡	Very rare
Amplification§	30%	15%	Very rare
Variant III mutation	5%¶	Very rare	Very rare
HER2			
Kinase domain mutation	Very rare	4%	Very rare
Amplification	2%	6%	Not known
ALK fusion‖	Very rare	7%	Not known
MET			
Mutation	12%	14%	13%
Amplification	21%	20%	Not known
TITF-1 amplification	15%	15%	Very rare
p53 mutation	60 to 70%	50 to 70%‡	75%
LKB1 mutation	19%	34%	Very rare
PIK3CA			
Mutation	2%	2%	Very rare
Amplification	33%	6%	4%

* Non–small-cell lung cancer includes squamous-cell carcinoma and adenocarcinoma.
† Neuroendocrine fields have been detected only in tissue surrounding tumors and have been characterized by extremely high rates of allelic loss and by c-MET overexpression (Salgia R: personal communication).
‡ Variations are based in part on smoking profiles.
§ The percentages include increased gene copy numbers from amplification or polysomy and represent percentages from resected cancers. The percentages are higher in primary tumors from patients with metastatic disease. Increased copy numbers have been reported in squamous dysplastic lesions but not in adenocarcinoma precursors.
¶ Genomic *EGFR* variant III mutations have been detected only in lung squamous-cell carcinoma, and these tumors are sensitive preclinically to irreversible EGFR tyrosine kinase inhibitors. The incidence of 5% is substantially lower than that of 30 to 40% for the detection in squamous-cell carcinoma or adenocarcinoma by immunohistochemical analysis or other techniques.
‖ The anaplastic lymphoma kinase *(ALK)* fusion gene (involving chromosome 2p), consisting of parts of *EML4* and *ALK,* is transforming in fibroblasts and occurs in adenocarcinoma but not in other types of non–small-cell lung cancer or other nonlung cancers.

Figure 4. Genetic abnormalities observed in lung cancer. Table originally published in (Herbst, Heymach et al. 2008**).**

Other Genetic Changes in Lung Cancer

Microsatellite alterations

Microsatellite repeats (also known as short tandem repeats) are highly polymorphic, widely distributed throughout the genome, and have been very useful markers for genetic mapping and analyzing small amounts of tumor samples for loss of heterozygosity (LOH). There are a handful of publications that reported microsatellite alterations (MA) in lung cancer (Merlo, Mabry et al. 1994; Shridhar, Siegfried et al. 1994; Adachi, Shiseki et al. 1995; Fong, Zimmerman et al. 1995; Ryberg, Lindstedt et al. 1995; Chen, Stroun et al. 1996; Miozzo, Sozzi et al. 1996). Overall, compiling the data from all the studies, 35% (37/106) of SCLCs and 22% (160/727) of NSCLCs have shown some examples of microsatellite alterations at individual loci. It also remains to be seen whether MA, observed in lung cancer plays a causative role resulting in disruption of specific genetic targets involved in carcinogenesis, or alternatively, reflects non-specific genome destabilization. Finally, it will be important to determine whether the detection of this defect and the resultant MA phenotype can be exploited for the early diagnosis of lung cancer.

Microsatellite alteration (MA) in lung cancer			
	Loci[a]	Small cell lung cancer	Non-small cell lung cancer
Peltomaki [258]	7	n.d.	2/87(2%)[b]
Mao [259]	9	5/10 (50%)	2/23 (9%)
Merlo [260]	34	15/33 (45%)	n.d.
Shridhar [261]	16	n.d.	13/38 (34%)
Ryberg [262]	5	n.d.	29/137 (21%)
Fong [263]	6	n.d.	7/108 (6%)
Adachi [264]	11	0/37 (0%)	16/55 (29%)
Chen [265]	3	16/21 (76%)	n.d.
Miozzo [269]	4	n.d.	25/51 (49%)
Hurr [266]	8	1/5 (20%)	3/38 (8%)
Rosell [267]	8	n.d.	24/35 (69%)
Sekine [268]	6	n.d.	8/27 (30%)
Lindstedt [270]	17	n.d.	31/128 (24%)
Total		37/106 (35%)	160/727 (22%)

[a]Number of loci (microsatellite markers) used. Some of the studies did not use all markers for every paired sample.
[b]The number of patients with MA (scored when at least one marker shows abnormality) among total lung cancer patients and percentage of abnormal cases. n.d., not done.

Figure 5. Microsatellite alterations in lung cancer.

Table originally published in (Sekido, Fong et al. 1998).

Expression of Telomerase Activity

All human chromosomes ends (telomeres) contain tandem tracks of the hexameric nucleotide repeat TTAGGG. During normal cell division, telomere shortening occurs due to the loss of telomeric repeats causing an `end replication problem'. This process of progressive telomere shortening leads to senescence and thus governing normal cell 'mortality'. The length of terminal telomeric restriction fragments is altered in various type of tumors, including lung cancer. Telomere shortening was detected in 14 of 60 primary lung cancers, while two cases showed telomere elongation (Hiyama, Ishioka et al. 1995). About 100% of SCLC and 80-85% of NSCLC, were demonstrated to express high levels of telomerase activity potentially leading to cellular 'immortality' (Kim, Piatyszek et al. 1994; Hiyama, Hiyama et al. 1995; Albanell, Lonardo et al. 1997). Functionally, high telomerase activity was associated with increased cell proliferation

rates and an advanced pathologic stage in primary NSCLCs (Albanell, Lonardo et al. 1997). There has been evidence that the telomerase is deregulated in the carcinoma in situ, indicating its involvement in early stages of development of lung cancer (Yashima, Litzky et al. 1997).

Aberrant methylation in Lung Cancer

DNA methylation involves covalent modification at the fifth carbon position of cytosine residues within CpG dinucleotides, which tend to be clustered in so-called CpG islands around the 5´ ends of many genes. DNA methylation is necessary for proper embryonic development and appears to be associated with genetic repression. There are several possible theoretical ways by which methylation could affect gene expression. First, methylated CpG residues could interfere directly with the binding to specific transcription factors to DNA. Secondly, it can attract the repression factors to bind to the promoter, thus affecting gene expression. Thirdly, it can alter the chromatin structure and, thus repress the gene expression. In NSCLC, hypermethylation has been shown to specifically inactivate the p16INK4/CDKN2 gene (Merlo, Herman et al. 1995; Otterson, Khleif et al. 1995), whereas in SCLC, regional hypermethylation has been found at chromosome 3p, but the precise gene target is uncertain (Makos, Nelkin et al. 1992). In a large percentage of primary NSCLCs the expression of p16 protein is reduced but the frequency of somatic p16 mutations is considerably low. This observation suggests, p16 is mostly inactivated by promoter hypermethylation.

Clonal Evolution

Cancers evolve by a reiterative process of clonal expansion, genetic

diversification and clonal selection within the adaptive landscapes of tissue ecosystems.

Lung cancer is no exception to this rule. Changes in certain pro-inflammatory

interleukin-8 (IL8) and some DNA-repair genes occur in nonmalignant lung tissue of

smokers and patients with lung cancer, a finding consistent with diffuse tissue injury

(Franklin, Gazdar et al. 1997; Spira, Beane et al. 2004; Spira, Beane et al. 2007). These

changes probably precede epithelial clonal evolution. Patches of clonally related cells, or

clonal patches containing 40,000 to 360,000 cells, have been mapped in the lung (Park,

Wistuba et al. 1999). The size and number of subclones in a clonal patch are correlated to

the progression of esophageal adenocarcinoma (Maley, Galipeau et al. 2006). Some of

the early events in the development of non small-cell lung cancer include loss of

heterozygosity at chromosomal region 3p21.3 (site of RASSF1A, a member of the Ras

association domain family, and FUS1), 3p14.2 (FHIT, a fragile histidine triad gene),

9p21 (p16), and 17p13 (p53) (Wistuba, Mao et al. 2002). Surprisingly, all these genes

are tumor-suppressor genes. In lung cancer, the gatekeepers are inactivated first to let the

disease progress. Several abnormalities have been observed in the normal tissue

surrounding the non-small-cell lung tumors. They include clonal patches with

methylation of promoter regions of genes (epigenetic changes), p53 mutation, EGFR

mutation, c-Myc amplification, loss of heterozygosity and microsatellite instability (Park,

Wistuba et al. 1999; Tang, Shigematsu et al. 2005; Sato, Shames et al. 2007). They may

be at a greater risk of recurrence and a second primary tumor. Recently, studies in mice
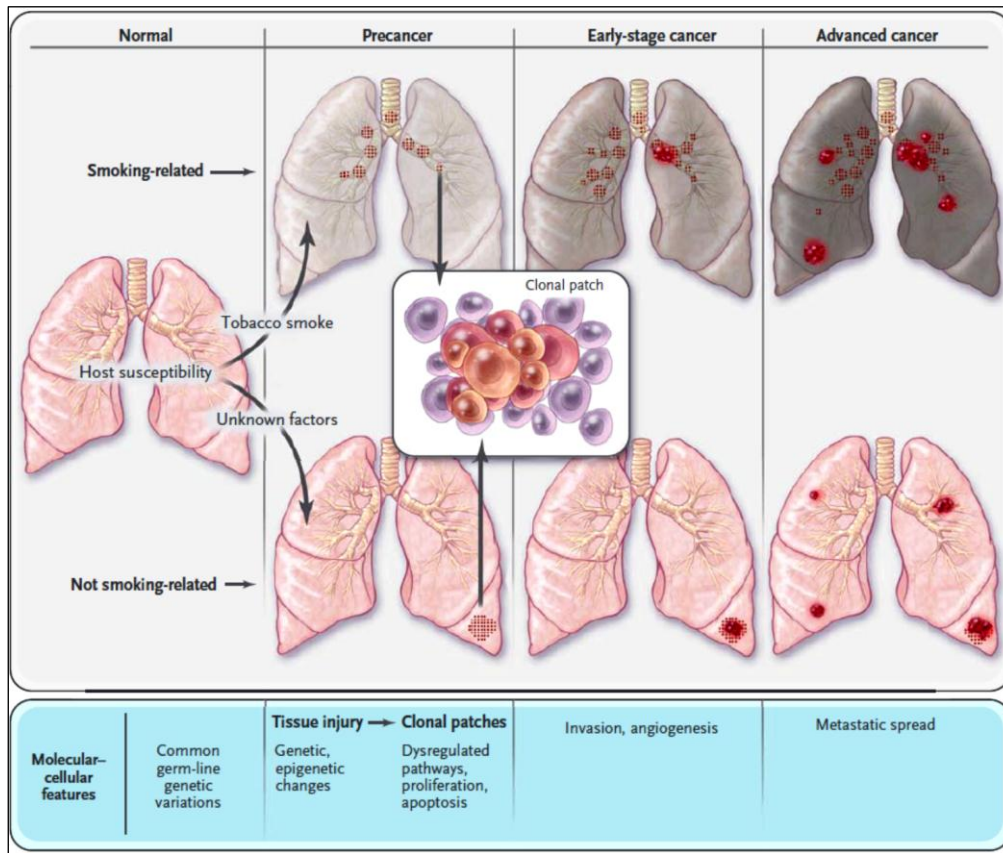
Figure 6. Molecular Evolution of Lung Cancer.

Environmental factors, such as tobacco smoke, and genetic susceptibility interact to influence carcinogenesis. Factors unrelated to smoking, including genetic, hormonal, and viral (e.g., human papillomavirus) have been suggested (Sun, Schiller et al. 2007). Many molecular changes in the earliest-stage of cancer also occur in the advanced disease (Zudaire, Lozano et al. 2008). Premalignant patches contain clones and subclones (inset), which can involve loss of heterozygosity, microsatellite instability, and mutations (e.g., in p53 and epidermal growth factor receptor). Smoking-related patches and primary cancers, usually squamous-cell carcinoma and small-cell lung cancer, most often develop in the central airway (Wistuba, Berry et al. 2000). Most tumors that are not related to smoking are adenocarcinomas and develop in the peripheral airways. Figure originally published in (Herbst, Heymach et al. 2008).

have identified the existence of bronchoalveolar stem cells, which are considered to be the precursors of lung adenocarcinomas (Kim, Jackson et al. 2005). The pathways implicated in proliferation of these stem cells are KRAS, Pten, Phosphoinositide 3-kinase and cyclin dependent kinase (Yanagi, Kishimoto et al. 2007; Yang, Iwanaga et al. 2008). The role of these stem cells with regards to lung tumorigenesis and prognosis need to be investigated.

Tobacco Smoking- the major cause of lung cancer

Smoking is directly responsible for approximately 90% of the lung cancers and is also strongly associated with cancer of the head and neck, esophagus and urinary bladder. Smoking accounts for atleast 30% of cancer deaths and is the single leading cause of cancer death in the United States. Figure 7 shows a the number of cancer related deaths attributed to smoking.
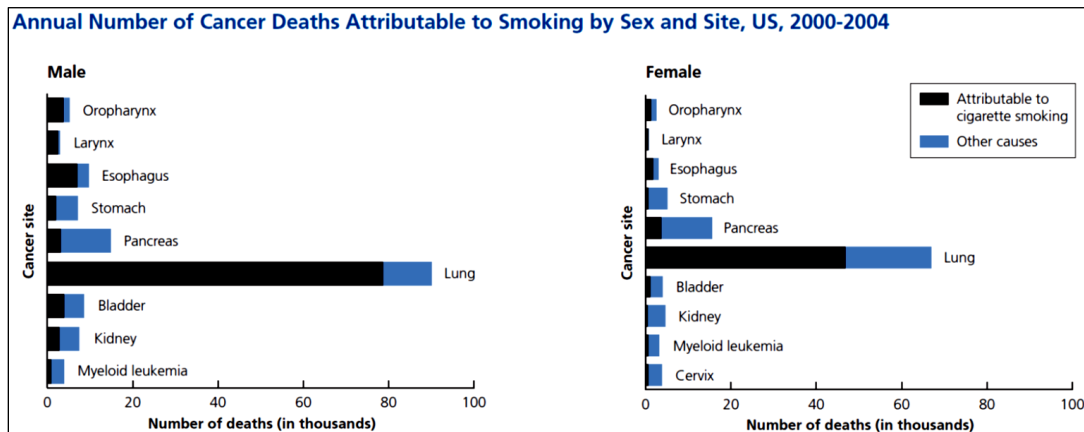
Figure 7. Cancer related deaths attributed to smoking. Figure originally published in (Siegel, Naishadham et al. 2012).

Smoking-specific lung cancer death rates

The lung cancer death rate increases exponentially with age in both current- and never-smokers. The absolute rates are 20-25 times higher among male smokers than never-smokers across most age groups, and 10-12 times higher in female smokers than never-smokers (Fig.8). As previously published in British Doctors' (Pike and Doll 1965; Doll and Peto 1978) and US Veterans' (IARC, 1986), these are studies on the relationship between age, smoking status and lung cancer deaths. As shown in Figure 8, the relationship between age groups when both age and lung cancer death are plotted on a log scale appears linear across most age groups. The linear relationship, however doesnot hold true in the oldest smokers (men and women aged >85 years), perhaps reflecting the combined effect of lower lifetime smoking in the oldest birth cohort and less complete ascertainment of lung cancer as a cause of death in the elderly.
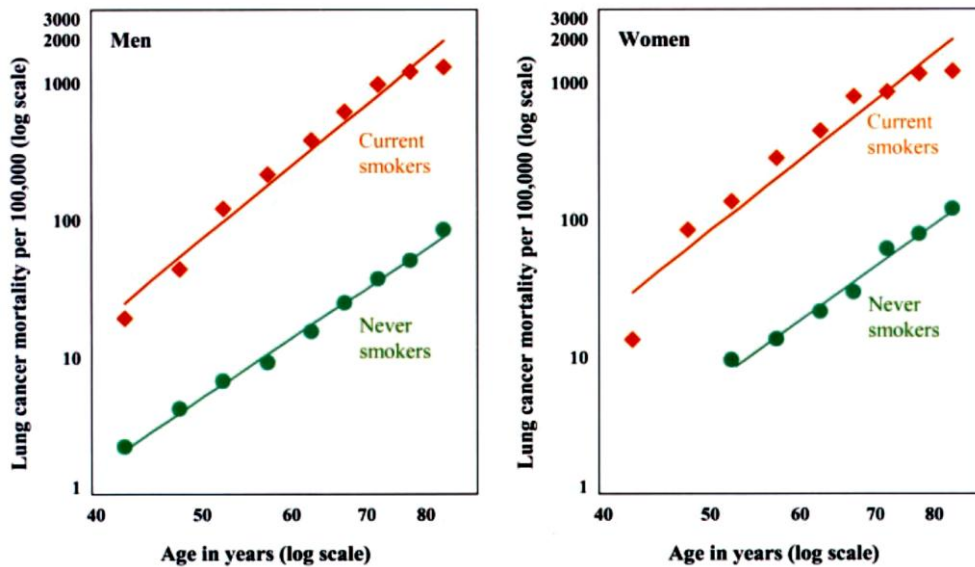
Figure 8. Lung cancer mortality by cigarette smoking status and age. Figure originally published in (Thun, Henley et al. 2002).

Trends in lung cancer risk after smoking cessation

For the first time, in its landmark publication, Peto et al.,2000 illustrated that the risk of lung cancer decreases when people stop smoking compared to those who continue to smoke. This also serves as an important piece of the evidence for causality effect of smoking. It also indicates that continuing exposure affects even the late stages of carcinogenesis. Figure 9 depicts the relationship between age and the cumulative probability of death from lung cancer according to smoking status in CPS-II from 1984 ± 91. In both men and women, the age-related increase in lung cancer risk is lowest in people who have never smoked, intermediate in those who have quit at various ages, and highest in current smokers. Among former smokers, the age-related increase is lower, the earlier the age of quitting. As seen in Figure 9, the cumulative probability of death from lung cancer continues to increase with age, even in never-smokers and former smokers. However, the increase in the probability of death is substantially slower than in current smokers. Studies that measure relative rather than absolute risk found that the relative risk of lung cancer approaches unity with time since cessation in former smokers, compared with those who have never smoked (US Department of Health and Human Services, 1990). The relative risk estimates fall below unity with time since cessation in former smokers compared to continuing smokers.
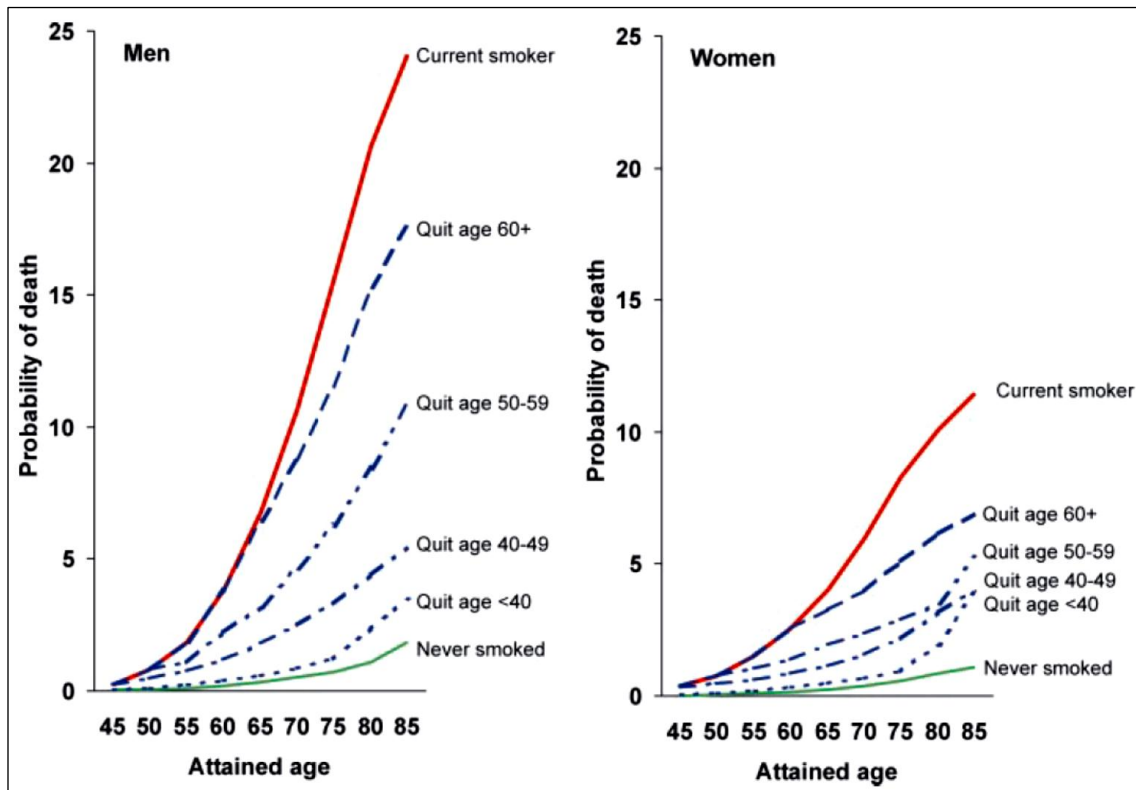
Figure 9. Probability of death from lung cancer: Cancer Prevention Study-II men and women, 1984 ± 1991.

It is based on nine rather than 6 years of follow-up to increase the statistical precision of the estimates, and excludes the first two years of follow-up (1982 ± 83) to avoid bias from smoking cessation due to tobacco-attributable disease. Figure originally published in (Thun, Henley et al. 2002).

Tobacco Carcinogens and Tobacco-Induced Lung Cancer

The 2003 International Agency for Research on Cancer (IARC) monograph entitled 'Tobacco Smoke and Involuntary Smoking', part of a review on the evaluation of carcinogenic risks to humans, came to the following conclusion regarding lung cancer on the basis of an extensive evaluation of the international literature (Hecht 2003). Cigarette smoking increases the risk of all histological types of lung cancer, including squamous - cell carcinoma, small-cell carcinoma, adenocarcinoma (including bronchiolar/alveolar carcinoma) and large-cell carcinoma. It might seem obvious that carcinogens present in the tobacco cause the numerous cancers that are associated with the use of tobacco products, but in this age of cancer genes, protein complexes, cellular circuitry and signal-transduction pathways, this simple fact is sometimes overlooked. Tobacco use is by far the most widespread link between exposure to known carcinogens and death from cancer, and is therefore a model for understanding mechanisms of cancer induction.

Carcinogens in tobacco products

Tobacco smoke is a heterogeneous mixture that contains approximately 4000 chemical compounds. Figure 10 below summarizes our knowledge of carcinogens in tobacco products (Hoffmann, Adams et al. 1987; Hoffmann, Djordjevic et al. 1995; Swauger, Steichen et al. 2002).More than 60 known carcinogens have been detected in the smoke that is emitted at the mouth end of the cigarette during puffing also known as mainstream smoke and most of the same carcinogens are present in the smoke that is emitted from the burning cone between puffs, which comprises most environmental tobacco smoke also known as sidestream smoke. All of the carcinogens in Table3 have

been formally evaluated by the IARC. In each case, studies in either laboratory animals or humans have provided sufficient evidence of carcinogenicity. There are also likely to be other carcinogens that might be related to these, but have not been fully characterized or evaluated. There is a vast range of potencies and concentrations among these carcinogens. In general, strong carcinogens; the carcinogen that reproducibly produces tumors in laboratory animals after treatment with relatively low doses (typically micrograms or milligrams), such as polycyclic aromatic hydrocarbons (PAHs), nitrosamines and aromatic amines, occur in smaller amounts (1-200 ng per cigarette) than weak carcinogens that produce tumors in laboratory animals only after administration of relatively high doses such as acetaldehyde (nearly 1 mg per cigarette). The total amount of carcinogens in cigarette smoke adds up to 1-3 mg per cigarette (similar to the amount of nicotine, which is 0.5-1.5 mg per cigarette), although most of this consists of weaker carcinogenic agents such as acetaldehyde, catechol and isoprene (Hecht 2003).

Tobacco smoke		
Chemical class	**Number of compounds‡**	**Representative carcinogens and typical amounts in mainstream smoke (ng per cigarette)§**
PAH‖	14	BaP	9
		Dibenz[*a,h*]anthracene	4
Nitrosamines	8	NNK	123
		NNN	179
Aromatic amines‖	12	4-Aminobiphenyl	1.4
		2-Naphthylamine	10
Aldehydes	2	Formaldehyde	16,000
		Acetaldehyde	819,000
Phenols	2	Catechol	68,000
Volatile hydrocarbons	3	Benzene 1,3-Butadiene	59,000 52,000
Nitro compounds	3	Nitromethane	500
Other organic compounds	8	Ethylene oxide Acrylonitrile	7,000 10,000
Inorganic compounds	9	Cadmium	132
Total	**61**		

Figure 10. List of carcinogens present in Tobacco smoke.

Table originally published in (Hecht 2003).

Mechanism of Chemical Carcinogenesis

When chemical carcinogens are internalized by cells, they are often metabolized. The initial step during conversion of organic xenobiotics into hydrophilic and excretable derivatives is mainly catalysed by CYP enzymes. CYP1A1 is the most important form in human lung. The resulting metabolic products are either excreted or retained by the cell. Inside the cell, carcinogens or their metabolic products can either directly or indirectly affect the regulation and expression of genes involved in cell-cycle control, DNA repair, cell differentiation or apoptosis. Some carcinogens act by genotoxic mechanisms, such as forming DNA adducts or inducing chromosome breakage, fusion, deletion, mis-segregation, and non-disjunction. For example, carcinogenic ions or compounds of nickel, arsenic and cadmium can induce structural and numerical chromosome aberrations(Kawanishi, Hiraku et al. 2002; Kasprzak, Sunderman et al. 2003). Others act by non-genotoxic mechanisms such as induction of inflammation, immunosuppression, formation of reactive oxygen species, activation of receptors such as arylhydrocarbon receptor (AhR) or oestrogen receptor (ER), and epigenetic silencing. Together, these genotoxic and non-genotoxic mechanisms can alter signal-transduction pathways that finally result in hypermutability, genomic instability, loss of proliferation control, and resistance to apoptosis some of the characteristic features of cancer cells. A comprehensive picture of the genotoxic and non-genotoxic mechanisms induced by tobacco carcinogens is shown in Fig.11.
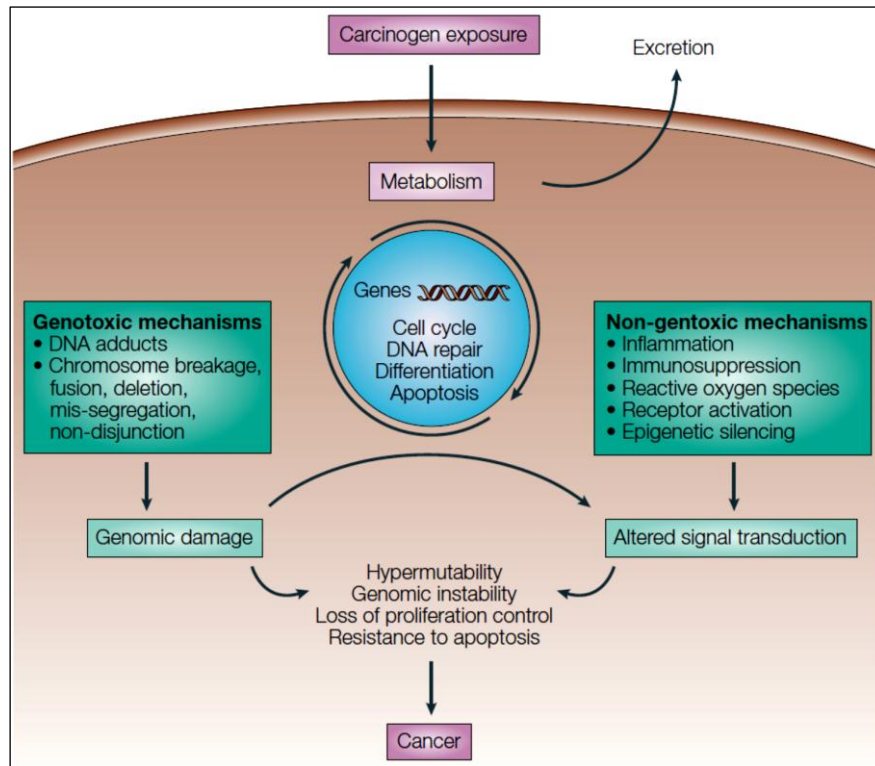
Figure 11.   An overview of genotoxic and non-genotoxic effects of carcinogens.

Figure originally published in (Luch 2005).

Tumor promotion and tumor initiation by tobacco carcinogens

Genotoxic carcinogens present in the cigarette smoke can induce damage in tumor suppressors or oncogenes in different ways, all of which contribute to the transformation of normal cells into tumor cells — this is known as the 'tumor initiation' stage in carcinogenesis. On the other hand, some chemical carcinogens present in the cigarette smoke are also capable of promoting the outgrowth of those transformed cell clones and of contributing to the generation of visible tumor cell masses — this is known as the 'tumor promotion' stage in carcinogenesis (Fig. 12). A large-scale series of experiments were undertaken to examine the promoting activity in cigarette smoke condensate by painting mouse skin which had been given a threshold dose of Benzopyrene. The concentration of Benzopyrene was considered too low for complete carcinogenesis (Roe, Salaman et al. 1959). However, it was thought that it could act as an initiator, especially when with other PAHs in the condensate such as BA and DBA that are negative or borderline as carcinogens but are relatively potent initiators (Van Duuren, Sivak et al. 1970). The factors shown in the figure below are only a few examples of a great number of factors that have been shown by gene-expression analysis to be altered following carcinogen exposure. In the figure below, binding of TCDD or BP to AhR leads to activation and translocation of the complex into the nucleus where it binds to xenobiotic-responsive elements (XREs) and induces the expression of a variety of different genes involved in carcinogen metabolism, including CYP forms 1A1, 1B1 and 1A2. It also changes the expression pattern of several factors involved in cellular growth and differentiation, such as plasminogen-activator inhibitor type 1 (PAI1), metallothionein II (MT-II), human enhancer of filamentation 1 (HEF1), guanine nucleotide exchange factor

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(GEF), COT and KRAS. Pro-apoptosis factors such as tumour-necrosis factor (TNF; superfamilies) and heat-shock protein 40 (HSP40) are downregulated, and cell-cycle genes can either be upregulated (such as cyclin B2) or downregulated (such as NEK2). COT and NEK2 are serine/threonine kinases (Luch 2005).
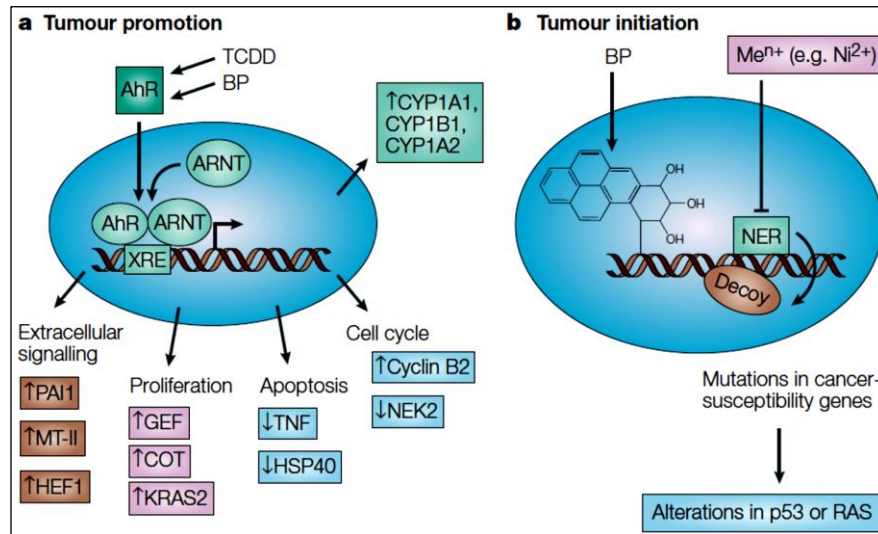
Figure 12. Tumor promotion and Tumor initiation by the carcinogens

a. Chemical compounds such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or benzopyrene (BP) result in tumor promotion through arylhydrocarbon receptor (AhR)-mediated signal transduction. b. Tumor initiation occurs through DNA-adduct-derived mutations in cancer susceptibility genes. DNA binding by genotoxic carcinogens such as activated BP leads to the induction of base pair or frame shift mutations in cancer-susceptibility genes such as TP53 or RAS. Figure originally published in (Luch 2005).

Co-carcinogens in Cigarette Smoke

Although there is a large body of information on promoters and their mechanism of action, the promotional procedure of a single application of a PAH carcinogen followed by multiple applications of promoters. One problem to this approach is that when a person smokes the person does not consume one or two components; rather they are exposed to the many components of cigarette smoke. This approach does not correctly simulate the simultaneous, long-term exposure of smokers to the many components of cigarette smoke. The much less studied procedures of co-carcinogenesis in which carcinogen and accessory substances are applied simultaneously over extended periods of time is a closer fit to the extended exposure of human smoking. Twenty-one compounds were tested for co-carcinogenic activity, 18 of which are found in cigarette smoke condensate (Van Duuren, Katz et al. 1973; Van Duuren and Goldschmidt 1976). Seven of the compounds were strong co-carcinogens that occur in cigarette smoke condensate (Van Duuren and Goldschmidt 1976) (Fig. 13).

Type of co-carcinogen	Carcinogen (BP 5μg)	Co-carcinogen (dose)	Mice with papillomas	Total papillomas	Squamous carcinomas
Controls	–	Acetone	0	0	0
	+	Acetone	14	16	10
Phenols	–	Catechol (2 mg)	1	1	1
	+	Catechol (2 mg)	36	90	31
	–	Pyrogallol (5 mg)	0	0	0
	+	Pyrogallol (5 mg)	40	95	33
Aliphatic hydrocarbon	–	Decane (25 mg)	1	1	0
	+	Decane (25 mg)	44	105	41
Aromatic hydrocarbon	–	B(e)P (15 μg)	0	0	0
	+	B(e)P (15 μg)	33	79	27
Phorbol ester hydrocarbon	–	TPA (2.5 μg)	5	5	0
	+	TPA (2.5 μg)	25	58	15

Figure 13. Co-carcinogenesis by some representative constituents in cigarette smoke condensate. Table originally published in (Van Duuren and Goldschmidt 1976). 5μg BP was applied in the same solution as the co-carcinogen in 0.1 ml acetone three times weekly to the dorsal skin of 50 female Swiss mice for 368 or 440 days. Controls of BP or co-carcinogen alone in acetone, or acetone alone, were similarly applied.

Mechanisms of Lung Carcinogenesis induced by smoking

The central role of DNA adducts: From Figure 14 we can see that carcinogens form the link between nicotine addiction and lung cancer, and DNA adducts are crucial in this process (Hecht 2003). Other tobacco related cancer follow a similar schemes, they may only differ in specific details. Most carcinogens in tobacco products require metabolic activation before they are able to react with DNA, although some, such as ethylene oxide, formaldehyde and acetaldehyde, can react directly. Metabolic activation is generally initiated by cytochrome P450 enzymes (P450s), which are part of normal mammalian systems designed to respond to foreign compounds (Guengerich 2001). Most of the metabolites formed in these P450-catalysed reactions are excreted in a detoxified form or undergo further detoxification reactions, but some metabolites are electrophilic and react with DNA to form DNA adducts. There is convincing evidence for the presence

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of adducts derived from these carcinogens in the DNA of human lung cells (Phillips 2002). There is also ample literature about the metabolic activation pathways that lead to DNA-adduct formation, as well as the competing detoxification pathways that lead primarily to excretion. Cellular repair systems remove DNA adducts through various DNA repair mechanisms and return DNA structure to its normal state (Wei, Cheng et al. 2000;Norbury and Hickson 2001; Goode, Ulrich et al. 2002). However, if the adducts persist and escape repair, mutations arise. It has been established conclusively that DNA adducts derived from cigarette-smoke carcinogens mainly Benzopyrene, cause miscoding most frequently in G-T and G-A mutations (Loechler, Green et al. 1984;Singer and Essigmann 1991; Seo, Jelinsky et al. 2000). If these permanent mutations occur in the crucial regions of the oncogenes (Ras and Myc), or in tumor-suppressor genes (p53 and CDKN2A (which encodes p16)), the result can be loss of normal cellular homeostasis and finally development of cancer (Osada and Takahashi 2002). Interference with the formation of DNA adducts results in decreased carcinogenicity (Adriaenssens, White et al. 1983; Hecht 1998; Kensler, Groopman et al. 1999; Boysen, Kenney et al. 2003). The central role of DNA adducts in carcinogenesis has been established and confirmed as biological fact over the past 50 years(Miller 1994).

The effect of Nicotine: More recent studies indicate, however, that additional deleterious effects of nicotine and tobacco specific-nitrosamines such as NNK might be mediated through cell-surface receptors. Nicotine, NNK, and probably NNAL, bind to nicotinic-acetylcholine receptors and other receptors, leading to activation of the serine/threonine kinase AKT (also known as protein kinase B), protein kinase A and other factors (Schuller 2002; West, Brognard et al. 2003). This leads to biological effects

such as decreased apoptosis, increased formation of arachidonic-acid metabolites, increased cell proliferation and thus, neoplastic transformation. Nicotine also stimulates angiogenesis and tumor growth (Heeschen, Jang et al. 2001) through nicotinic-acetylcholine receptors. The putative mechanism involves endothelial production of nitric oxide, prostacyclin and vascular endothelial growth factor. These data suggest that nicotine might have tumor-promoting or co-carcinogenic activity other than just contributing to addiction to cigarette smoking. However, mixed results have been obtained, depending on the animal model and study design used, with some studies showing evidence of co-carcinogenicity, whereas others showed no effect or inhibition by nicotine (Habs and Schmahl 1976; Gurkalo and Volfson 1982; Berger, Petru et al. 1987; Chen and Squier 1990).Carcinogenicity studies of nicotine have mostly been negative, except when nicotine was administered in the presence of hyperoxia, which caused some tumors in hamsters(Schuller, McGavin et al. 1995).

Effect of Co-carcinogens and tumor promoters: Tobacco smoke also contains co-carcinogens and weakly acidic tumor promoters (Van Duuren, Katz et al. 1973; Hecht, Carmella et al. 1981) (Fig. 10). Although the mechanisms by which tobacco-smoke co-carcinogens and tumor promoters enhance carcinogenesis are not clearly established, studies with other tumor promoters show that they can activate signaling by protein kinase C, activator protein 1 (AP1) and other factors(Dong, Birrer et al. 1994).

Another emerging mechanism of gene silencing induced by tobacco carcinogenesis is by promoter hyper-methylation (Baylin, Herman et al. 1998).Recent studies show that inactivation of the CDKN2A tumor-suppressor gene and O6-

methylguanine-DNA methyltransferase DNA-repair genes are common in non small-cell lung cancer(Palmisano, Divine et al. 2000).

An additional requirement is that this process has to continue on an average of 50 years. In other words, on an average a man develops lung cancer after smoking for 50 pack years, which is one pack per day for 50 years. The resulting chronic DNA damage is consistent with the multiple genetic changes that are seen in lung and other tumors, as normal tissues progress from hyperplasia to dysplasia to carcinoma in situ to invasive cancer(Park, Wistuba et al. 1999; Osada and Takahashi 2002; Wistuba, Mao et al. 2002).
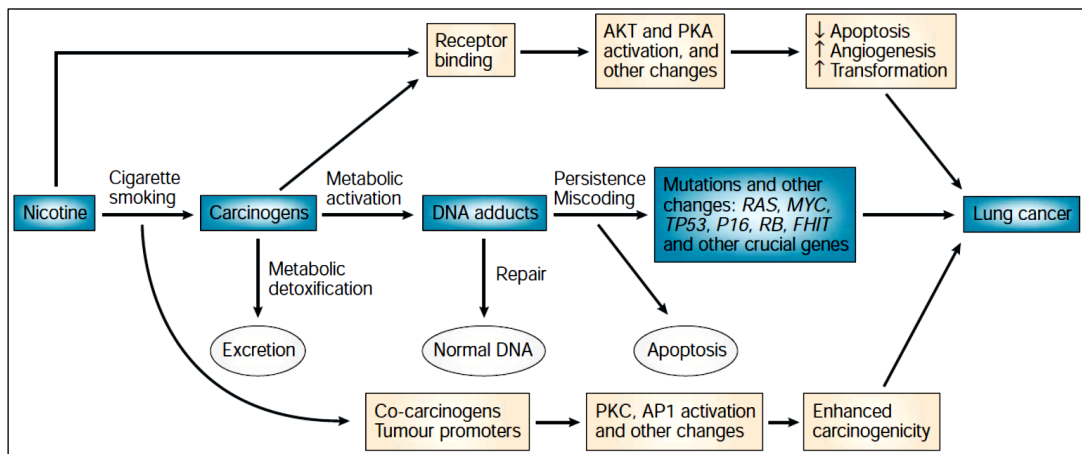
Figure14. Scheme linking nicotine addiction and lung cancer through tobacco-smoke carcinogens.

The events outlined in Figure must occur chronically for approximately 30-50 years for cancer to develop. Carcinogens are delivered with each puff of each cigarette every day during this time period. Figure originally published in (Hecht 2003).

The TGF-β pathway

The TGF-β super-family of growth factors comprises seven genes in Drosophila melanogaster and at least 30 genes in mammals, including 3 TGF-β isoforms, 4 activin β‑chains, the protein nodal, 10 bone morphogenetic proteins (BMPs) and 11 growth this receptor family, some of which may be shared by different TGF-β ligands. All these ligands are synthesized as dimeric pre-proproteins (Samanta and Datta 2012). Dimerization requires the pro-domains (Gray and Mason 1990; Shimmi, Umulis et al. 2005) and therefore occurs intra-cellularly, before cleavage by proteases of the subtilisin like pro-protein convertase (SPC) family (Dubois, Laprise et al. 1995; Constam and Robertson 1999). The mature, fully processed dimeric growth factors are subsequently secreted. TGFβs are secreted as latent forms while still non-covalently attached to their pro-peptide. They require a further activation step to release the active ligand (Annes, Munger et al. 2003), which involves the metallo-protease BMP1 (also known as Tolloid in D. melanogaster) (Ge and Greenspan 2006). Ligands of the TGF-β superfamily of growth factors regulate many cellular functions including cell growth, adhesion, migration, cell-fate determination and differentiation, and apoptosis. Malfunctions in signaling downstream of TGF-β are implicated in serious human diseases such as cancer, fibrosis, wound-healing disorders, and several hereditary conditions such as familial primary pulmonary hypertension and hereditary Haemorrhagic Telangiectasia (HHT). The growth inhibitory effect of TGF-β signaling in epithelial cells explains its role as a tumor suppressor in carcinomas, although TGF-β expression by tumor cells contributes to cancer progression as well. Signaling primarily occurs through Smad protein dependent pathways whereby ligand binding to TBRII induces phosphorylation and activation of

TGF-β type I receptor (TβRI). After interaction with TβRI, phosphorylated Smad2 and

Smad3 dissociate to form a heterotrimeric complex with Smad4 and translocate into the

nucleus to regulate gene transcription (Fig. 15). TGF-β signaling may also proceed via

less well-understood SMAD independent pathways. These "noncanonical" pathways

involve various signaling cascades including Ras/ERK, Rho/ROCK, and TAK1/MAPK

and are likely to have important roles in mediating the pro-tumorigenic effects of

TGFβ(Massague 2000). Depending on context, TGF-β signaling may alternatively

function to suppress tumor growth or to promote tumor cell invasion and metastasis.
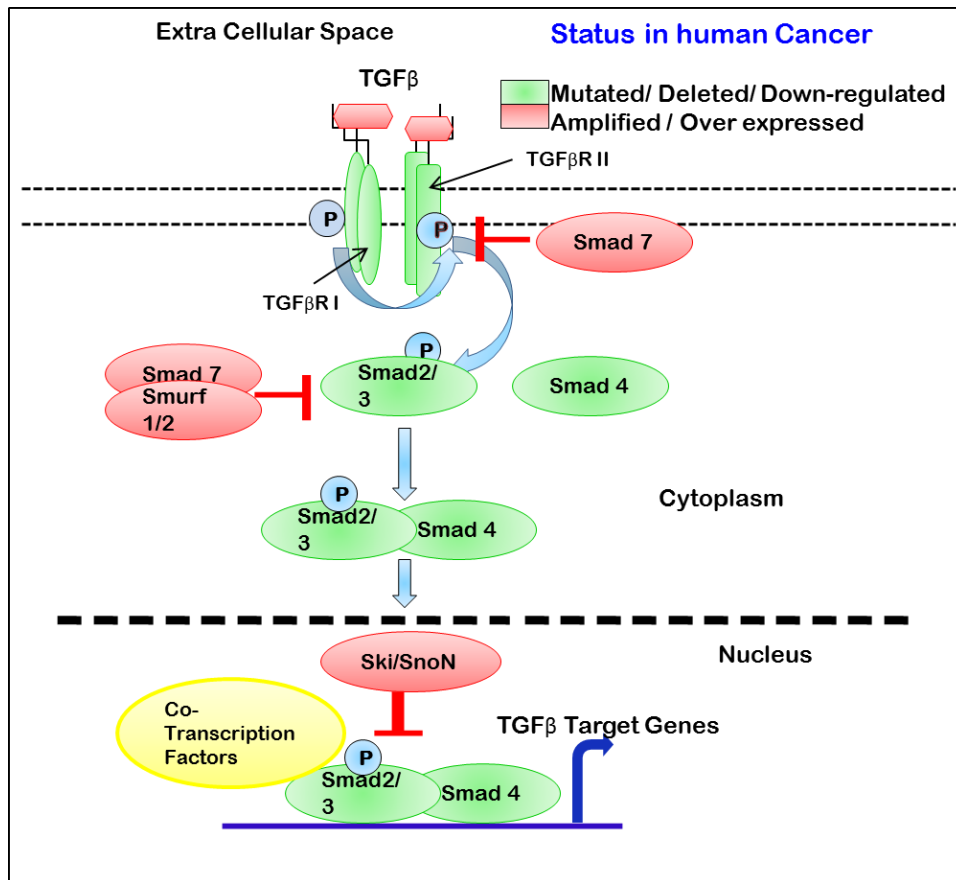
Figure15. Alterations of the Smad dependent TGF-β pathways in Cancer.

The components that are mutated, deleted or down-regulated, are shown in green, while the components that are amplified or over-expressed, are shown in red. Figure originally published in (Samanta and Datta 2012).

Lung cancer and TGF-β

Although most lung cancer cells secrete TGF-β, the malignant transformation in lung cancer results in a loss of the tumor suppressor effects of TGF-β. Loss of TGF-β response, which results in loss of inhibitory effect of TGF-β on proliferation, has been associated with tumor development and or tumor progression in several cancers(Kim, Im et al. 2000). Reduced expression and inactivation of TGF-β receptors were associated with loss of sensitivity with anti-proliferative effects of TGF-β in carcinogenesis(de Caestecker, Piek et al. 2000). The unresponsiveness to TGF-β could be caused by multiple ways involving both genetic and epigenetic alterations of TGF-β type II receptor expression (TβRII)(Osada, Tatematsu et al. 2001). Mutations within the coding sequence of the TβRII gene are rare in non–small cell lung cancer (NSCLC). Mutations in Smad2 and Smad4 genes have been found in 5-10% of lung cancers (Nagatake, Takagi et al. 1996; Uchida, Nagatake et al. 1996). It was shown that 29 of 33 lung cancer cell lines are unresponsive to TGF-β–induced growth inhibition (Hougaard, Norgaard et al. 1999). TβRII expression was shown to be decreased in 80% of squamous cell carcinoma, 42% adenocarcinoma, and 71% large cell carcinoma (Anumanthan, Halder et al. 2005). Study from our lab had shown that the stable expression of TβRII in TGF-β–unresponsive cells restores TGF-β–induced inhibition of cell proliferation, induction in apoptosis, and decrease in tumorigenicity(Anumanthan, Halder et al. 2005). The loss of TGFβRII not only contributes to tumorigenesis but also contributes to metastasis. In a recent study, researchers have identified a transcriptional profile that distinguishes invasive from noninvasive lung adenocarcinoma, this includes reduced expression of a previously identified tumor suppressor, TGFβRII, and suggests that down regulation is an early step

in adenocarcinoma metastasis. Potential mediators of invasion resulting from suppression of TGFβRII in tumors were identified and include several transcriptional factors and E-cadherin, which were lower in TGFRII knock-down cells and in primary invasive tumor specimens(Borczuk, Kim et al. 2005). However, there is a conflicting observation that in lung cancer, overexpression of TGF-β is associated with better prognosis in 5-year patient survival (Inoue, Ishida et al. 1995). TGFβ-1 level in serum was elevated after radiotherapy in lung cancer, and the risk of radiation induced lung injury is associated with TGF-β1 single nucleotide polymorphism(Yuan, Liao et al. 2009; Zhao, Wang et al. 2009). Additional complexity for the involvement of the TGF-β pathway is provided by the feedback loop exhibited by the inhibitory Smads namely, Smad6 and Smad7. In a recently publication (Jeon, Dracheva et al. 2008) observed that in lung cancer, Smad6 is overexpressed in a portion of the tumors, and high expression of Smad6 is associated with poor survival in patients with non-small cell lung cancer. Knockdown of Smad6 in overexpressed lung cancer cells induced apoptosis and growth arrest at the G1 phase, which contributed to the reestablishment of TGF-β homeostasis in lung cancer cells by reactivating the TGF-β signal pathway(Jeon, Dracheva et al. 2008). This study indicates that I-Smads play a critical role in tumorigenesis by regulating TGF-β homeostasis in normal and cancer cells. Given the causal role of smoking in inducing lung cancer, there has been no report that smoking effects the TGF-β signaling in the context of lung cancer or infact in any cancer. So we wanted to pursue the effect of long-term smoking on TGF-β signaling and what effect does it have with regards to lung tumorigenesis and lung cancer therapy.

The aims of the dissertation were:

1. **To evaluate the role of long-term smoking on Smad dependent TGF-β signaling and its contribution to lung tumorigenesis in NSCLC.**

   Epidemiological studies have demonstrated that most cases of lung cancers (85-90%) are directly attributable to cigarette smoking. Although much information has been gained about the effects of cigarette smoking on various signaling pathways causing lung cancer, nothing is known about the effect cigarette smoking on the TGF-β-induced tumor suppressor function in lung cancer. To address this issue, lung adenocarcinoma A549 and immortalized bronchial epithelial HPL1A cells were chronically treated with cigarette smoke condensate (CSC) and Dimethyl sulphoxide (DMSO, as a control) to mimic the conditions of long-term cigarette smoking. A possible role for long-term smoking in decreasing Smad3 expression; abrogating TGF-β mediated growth inhibition and thus its contribution to lung tumorigenesis was identified.

2. **To evaluate the role of long-term smoking mediated decrease in Smad3 to resistance in Carboplatin based chemotherapy in NSCLC.**

   While numerous cell-signaling pathways are known to play decisive roles in chemotherapeutic response, relatively little is known about the impact of the Smad dependent TGF-β pathway on therapeutic outcome. Previous reports suggested that lung cancer patients who continue to smoke while receiving chemotherapy have poorer outcome than their nonsmoking counterparts. In our

previous study, we showed that long-term CSC mediated downregulation of Smad3 induces tumorigenesis. The objective of this study was to study identify the role of Smad3 in chemoresistance induced by cigarette smoke condensate (CSC) in human lung cancer cell lines namely, A549 and HPL1A.

CHAPTER II

Materials and Methods

Cell lines and Cell Culture

Human lung adenocarcinoma cells (A549) obtained from ATCC and human lung immortalized epithelial cells (HPL1A) (a kind gift of Dr. T. Takahashi), Mycoplasma negative by a PCR detection method (4/10/10 and 10/08/10 testing Sigma Venor-Gem); were maintained in RPMI with 10% FBS supplemented with penicillin/streptomycin and L-glutamate. All cell lines were cultured and maintained at 37ºC in a humidified incubator in the presence of 5% CO_2. Cells were treated with CSC at 25µg/ml or with the same volume of DMSO as controls for 4,100 and 300 days Mycoplasma negative by a PCR detection method (4/10/10 and 10/08/10 testing Sigma Venor-Gem). Both A549 and HPL1A were obtained during 2006, were tested and authenticated by DNA profiling for polymorphic short tandem repeat (STR) markers at Vanderbilt University DNA Sequencing & Analysis Core most recently in April 2011.

Reagents and antibodies

CSC was purchased from Murty Pharmaceuticals, Inc. It was prepared from the University of Kentucky's 3R4F cigarettes. G-Sepharose beads were purchased from Sigma Bio chemicals (St. Louis, MO). TGF-β1 was purchased from R&D Systems (Minneapolis, MN). The anti-β-actin antibody, Bcl-2 siRNAs, N-ter was purchased from Sigma Biochemicals (St. Louis, MO). Rabbit anti-Smad2 and anti-Smad3 were from Zymed Laboratories, Inc. (San Francisco, CA). Mouse anti-Smad3, anti-Smad4 and anti-Bcl-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-

Smad2, Bax, Bcl-xl, and Bcl-w antibodies were purchased from Cell Signaling Technology (Beverly, MA). MTT kit and ChIP assay kits were purchased from Millipore (Temecula, CA).

Immunoprecipitation and Immunoblot Analyses

For immunoprecipitation, cells were serum-starved for 2 hours and treated with 12.5ng/ml TGF-β1 for 1 hour and cell lysates were made. 4, 100 and 300-days of CSC treated A549 and HPL1A cells were serum-starved for 2 hours and treated with 12.5 ng/ml TGF-β1 for 1 hour. Cells were solubilized in lysis buffer. An equal amount of each protein lysate was incubated with both anti-Smad2 and anti-Smad3 polyclonal antibodies for 2 hours at 4°C, followed by incubation with 20 µl of protein G-Sepharose beads for 1 hour. The immune complexes were analyzed by Western blot analyses with mouse anti-Smad4 antibody. Protein lysates were also subjected to Western blot analyses with anti-phospho-Smad2, anti-Smad2, anti-Smad3, anti-Smad4 and mouse anti-β-actin antibodies.

Transcriptional response assay

Cells were transiently transfected with either p-3TP-Lux, (CAGA)$_9$-MLP-Luc or Smad3 plasmids and with CMV-β-gal plasmid as an internal control for transfection efficiency. . The Smad3 Luc-reporter plasmid containing 1,892-bp fragment corresponding to -1879 to +13 relative to the ATG start site of the human gene Smad3 cloned upstream of the luciferase-reporter gene was a generous gift of Dr. Thomas J. Kelly (Department of Pediatrics, Case Western Reserve University, Cleveland, US). For the experiments with p-3TP-Lux, (CAGA)$_9$-MLP-Luc, transfected cells were incubated in 0.2% FBS with 5 ng/ml TGF-β1 for 22 hours. For experiments with the Smad3

promoter they were not treated with TGF-β1. Cell lysates were used to measure both luciferase and β-gal activities, and the normalized luciferase activity was presented.

Quantitative Real Time PCR

Total RNA was isolated from cells by extraction of RNA by using Trizol reagent (Invitrogen) and alcohol precipitation. The results would be quantitated by $2^{\wedge}(-\Delta\Delta Ct)$ method where $\Delta\Delta Ct = \Delta Ct,CSC - \Delta Ct,DMSO$. Here, ΔCT, CSC is the Ct value for Smad3 normalized to the GAPDH in 300 day CSC treated cells and ΔCt, DMSO is the Ct value for the Smad3 also normalized to GAPDH in 300 days DMSO treated cells. Fold changes were calculated in paired samples of CSC and DMSO treated for various time points using the Sybr green real-time PCR method and the Applied Bio systems 7700 Sequence, Detection System. Reaction conditions were 95°C for 10 sec, 58°C for 45 sec, (45 cycles).

Primers and probes used for RT-PCR were as follows:

Smad3 Forward: 5'- CCA GAA CCA AAC CTC AAC AC-3'

Smad3 Reverse: 5'- TCCTCTTGCGTACGTTTTTC-3'

GAPDH Forward: 5'- GCCTCAAGATCATCAGCAATG-3'

GAPDH Reverse: 5'- CTTCCACGATACCAAAGTTGTC-3'

Cell Viability Assay

Cell viability was measured with the MTT assay according to manufacturer's recommendations. Cells in the exponential phase were dispensed in 96-well plates

overnight at a density of 2×10^3 cells per well. Cells were then treated with TGF-β

(5ng/ml) for a total of 5 days. TGF-β containing media was replaced every other day. The

optical density was transformed to cell numbers by a standardization curve done

simultaneously.

Cell Death ELISA

Various time points of CSC and corresponding control cells (20,000 per well)

were seeded into 12-well plates and allowed to attach for 20 hours. Cells were serum

starved for 72 hours. Cells (floating and adherent) were lysed in 200 µl of lysis buffer.

Each lysate (10 µl) was used for ELISA (Roche Molecular Biochemicals, Indianapolis,

IN).

Bcl2 siRNA knockdown

20nM of Bcl2 siRNAs (Sigma Aldrich) were transiently transfected into 300-days

CSC treated A549 and HPL1A cells using N-ter a transfection reagent (Sigma Aldrich)

according to the manufacturer's instructions. A control siRNA (nonhomologous to any

known gene sequence) and transfection just with the transfection reagent N-ter were used

as negative controls. The concentration was determined from the initial experiments

optimizing the concentration for the minimum concentration of siRNA required to

knockdown Bcl2 more than 80%. The level of Bcl2 expression was determined by

Western blot using a Bcl2 antibody, 72 hours after transfection. Three independent

experiments were conducted to determine specific silencing of the targeted Bcl2 gene.

Bcl2 siRNAs sequences are available upon request.

Apoptosis by Flow cytometry

The cells were transfected with Bcl2 siRNAs and then the cells were serum starved for 72 hours and assayed for apoptosis. The cells were harvested, washed with cold PBS, and cells were stained with PI and Annexin-V-FITC using ANNEXIN V-FITC/PI Kit (BD Biosciences) for apoptosis analysis according to the manufacturer's protocol. Stained cells were immediately analyzed by fluorescence activated cell sorting (FACS; Cell Lab Quanta SC; Beckman Coulter).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assay (ChIP) assays were performed via a commercially purchased chromatin immunoprecipitation kit (Millipore, Temecula, CA), using either anti-Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA; pep-2) or anti-HDAC1 (Millipore; 17-608), antibodies. Cells were first cross-linked for 10 min by adding formaldehyde directly to tissue culture medium to a final concentration of 1%. Cross-linked cells were then washed twice with cold PBS (with protease inhibitors), scraped, pelleted, resuspended in 200 µl SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0), and incubated for 10 min on ice. The lysates were then sonicated for five cycles of 30 s each, resting on ice for 1 min between cycles. After sonication, the samples were centrifuged and the supernatants diluted 10-fold in ChIP dilution buffer with protease inhibitors and precleared with 60 µl salmon sperm DNA/protein A Agarose-50% slurry for 60 min at 4°C. Cross-linked chromatin was incubated overnight with 5 µg Sp1, 4 µl HDAC1, or control IgG in a total volume of 1 ml at 4°C. Antibody-protein-DNA complexes were isolated by immunoprecipitation with 60 µl salmon sperm DNA/protein

A. After extensive washing, pellets were eluted by freshly prepared elution buffer (1% SDS, 0.1 M NaHCO3). Formaldehyde cross-linking was reversed by 5-12-h incubation at 65°C after adding 20 μl 5 M NaCl. Samples were purified through PCR purification kit columns (Qiagen, Chatsworth, CA) and used as a template in PCR. ChIP primers:

5′-CGAGGGGGCGGTGACAGCA-3′ and

5′-CCTCGCTAGCTTTGATGCGCAGA- 3′ were used to amplify a 200-base pair fragment corresponding to the core Smad3 promoter. Samples from at least three independent immunoprecipitations were analyzed.

Stable Overexpression of Smad3 in 300-day CSC treated cells

300-day CSC treated A549 cells were stably transfected with Smad3-pCDNA3 or pCDNA3 vector. Transfected cells were selected for two weeks in presence of G418, 400μg/ml for 300-day A549, and 200μg/ml for 300-day HPL1A cells. Polyclonal population cells were used to isolate clones and the expression of Smad3 in each clone was verified by immunoblot analysis using Smad3 antibody. Clones that expressed a higher level of Smad3 were selected for experiments and maintained in RPMI containing 10% FBS in the presence of 300μg/ml for A549 and 150μg/ml for HPL1A cells.

Stable Overexpression of Bcl2 in A549 and HPL1A cells

A549 and HPL1A cells were stably transfected with Bcl2-pCDNA3 or pCDNA3 vector. Transfected cells were selected for two weeks in presence of G418, 600 μg/ml for A549, and 400 μg/ml for HPL1A cells. Polyclonal population cells were used to isolate clones and the expression of Bcl2 in each clone was verified by immunoblot analysis

using Bcl2 antibody. Clones that expressed a higher level of Bcl2 were selected for

experiments and maintained in RPMI containing 10% FBS in the presence of 300 μg/ml

for A549 and 200 μg/ml for HPL1A cells.

Smad3 siRNA knockdown in Bcl2 overexpression clones

20 nM of Smad3 siRNAs (Sigma Aldrich) were transiently transfected into A549,

HPL1A Bcl2 overexpressing clones using N-ter, a transfection reagent (Sigma Aldrich),

according to the manufacturer's instructions. A control siRNA (nonhomologous to any

known gene sequence) and transfection just with the transfection reagent N-ter were used

as negative controls. The concentration was determined from the initial experiments

optimizing the concentration for the minimum concentration of siRNA required to

knockdown Bcl2, Smad3 more than 80%. The level of Smad3 expression was determined

by Western blot using Smad3 antibody, 72 hours after transfection. Three independent

experiments were conducted to determine specific silencing of the targeted Bcl2, Smad3

genes. Smad3 siRNAs sequences are available upon request.

MTT Assay

In a 96-well flat-bottomed plate, 2000 cells/100 μl of cell suspension were used to

seed each well. The cells were incubated overnight to allow for cell attachment and

recovery. Cells were treated with indicated drugs and incubated for 72 hours at 37°C.

After treatment, 10 μl of a 5 mg/ml solution in PBS of the 3-(4,5- dimethylthiazol-2-yl)-

2,5-diphenyltetrazolium bromide (MTT) tetrazolium substrate (Millipore, Billerica, MA)

was added to each well and incubated for ~4 hours at 37°C. The resulting violet

formazan precipitate was solubilized by the addition of 100 μl of a 0.04 M HCl in

Isopropanol solution. The plates were then analyzed on a microplate reader (MRX; Dynex Technologies, West Sussex, United Kingdom) at 570 nm and 630 (background) to determine the absorbance of the samples.

Cell cycle Analysis

Cells were plated at $1 \times 10^6/10$ cm dish and allowed to grow overnight, and subsequently treated with carboplatin (70 µM) for 48 hours. Single-cell suspensions were labeled with 50 µg/ml propidium iodide (Sigma), and approximately 10^6 cells in 1 ml were analyzed by flow cytometry. Ten thousand cells were evaluated, and the percentage of cells in the G2 phase was denoted by the 2N population phase, which was determined using FlowJo Software.

Tissue Samples and Tissue Microarrays

Unstained tissue sections from tissue microarrays were obtained from the Vanderbilt Lung SPORE. They consist of Paraffin-embedded formalin-fixed tissues obtained from the archives of the pathology department at Vanderbilt University and the Department of Veterans Affairs Medical Center (VAMC, Nashville, TN). The study was approved by the local Institutional Review Boards for all institutions involved. Tissue microarrays of NSCLCs were prepared from paraffin blocks following the methods described by Kononen and colleagues and reported earlier. There are 42 tumors represented on the arrays. Hematoxylin and eosin (H&E) stained sections from all tissue blocks were reviewed by a pathologist with expertise in lung cancer (AG).

DNA Laddering

Cells were serum-starved for 72 hours. Cells (floating and adherent) were collected and lysed. RNase A (5 µg/ml) was added to the clear lysate and incubated at 56°C for 2 hours. Proteinase K (200 µg/ml) was then added and incubated at 50°C for an additional 2 hours. Treated samples were extracted twice with TE-saturated phenol/chloroform. DNA was precipitated with cold ethanol, washed, suspended in 20 µl of TE buffer, and analyzed by agarose gel electrophoresis.

Soft agar assay

To test the effects of smoking on anchorage-independent growth, vector controls and three stable Smad3 clones were compared for clonogenic potential in semi-solid medium. 10^3 to 10^5 cells from each pool were suspended in 1 ml of 0.4% in sea plaque agarose containing 10% FBS medium and then plated on the top of 1 ml of semi-solidified 0.8% agarose in the same medium in 35-mm plates. Plates were incubated for 17 days at 37°C in the presence of 5% CO_2 in a humidified incubator. Colonies grown on soft agarose were counted by automated colony counter and pictures of colonies were taken under the inverted microscope.

Xenograft assay

Cells were assayed for tumorigenicity in 7-week-old athymic nude mice. $1X10^6$ cells were injected subcutaneously behind the anterior fore limb of each mouse as indicated. The animals were monitored for tumor formation twice a week. Tumors were measured by slide calipers and tumor volume was calculated by the equation: $V = L\ XW^2$

X 0.5, where V = volume, L = length, and W = width. Growth curves for tumors were plotted from the mean volume +/- SD of tumors from six mice.

Immunohistochemistry

Immunohistochemistry was performed as described in (19) with mouse monoclonal Smad3 incubated for 2 hours (dilution 1:100). Smad3 expression was evaluated semi quantitatively based on the intensity of staining and was scored as weak (+1), moderate (+2), and intense (+3). Samples with no staining were considered negative, and samples with weak-to-intense staining were considered positive.

Public Database analysis for expression of Smad3

We analyzed raw microarray data files from published NSCLC cohort microarray databases to assess the expression of the Smad3 in clinical samples of NSCLC. We used 361 adenocarcinomas from a recent multi-site blinded validation study by the Director's Challenge Consortium for the Molecular Classification of Lung Adenocarcinoma (22) that were initially surgically resected in Memorial Sloan Kettering (n=104), University of Michigan (n=178), and H. Lee Moffitt cancer center (n=79) (National Cancer Institute Cancer Array database, experiment ID 1015945236141280:1 (https://caarraydb.nci.nih.gov/caarray). We excluded adenocarcinomas from the Dana Farber institute within the same report because of their reduced total gene expression levels as well as samples that were excluded in their original report. All NSCLC samples analyzed included only those that passed the quality checks in the original published reports of the mentioned cohorts. In addition, each gene expression sample analyzed represents one unique patient. All raw data files were imported and analyzed using the

BRB-ArrayTools v.3.7.0 Beta Genes were normalized independently by cohort. Common genes present in all gene chip platforms (Affymetrix® HG-U133A, HG-U133 plus 2.0, and U95A) were identified using NetAffxTM http://www.affymetrix.com/analysis/index.affx. Kaplan-Meier and log-rank test survival analyses based on Smad3 expression were performed using the R 2.6.0 statistical package (http://www.r-project.org/). Expression of Smad3 was analyzed in the aforementioned cohort. Z-scores were calculated and plotted using Graph Pad Prism Software and Log-rank statistics were then performed as described earlier to measure the survival differences among the identified patients.

Statistical Analysis

Descriptive statistics including mean values and SD were calculated using Prism software (Graph pad, La Jolla, CA). All data are representative of at least three independent experiments and are expressed as the means ± SD unless otherwise indicated. ANOVA was used to assess the differences between experimental groups, and survival curves, unless otherwise indicated.

CHAPTER III

Introduction

Cigarette smoking is the main risk factor for lung cancer, accounting for 90% of cases in men and 70-85% of cases in women. Approximately 1.3 billion people smoke cigarettes worldwide, which contributes to 5 million preventable deaths per year (Schembri, Sridhar et al. 2009). The vast majority of lung cancers are strongly correlated with tobacco consumption, and smoking cessation remains the only known way of reducing cancer risk in smokers (Thomson, Fisher et al. 2004). Cigarette smoking has been found to induce a number of genetic and molecular changes in the respiratory tract, including cellular atypia, loss of heterozygosity (Powell, Klares et al. 1999), and promoter hyper methylation (Guo, House et al. 2004). Microarray studies of bronchial epithelial cells have indicated that cigarette smoke induces, primarily, the expression of xenobiotic-metabolizing and redox-regulating genes but also point to the effects on tumor suppressor genes, oncogenes, and genes involved in the regulation of inflammation (Spira, Beane et al. 2004). Systematic analysis of mRNA and protein expression levels among thousands of genes has also contributed to defining the molecular network of lung carcinogenesis. Despite the fact that enormous progress in the understanding the molecular mechanisms leading to lung cancer has been made, how intrinsic Smad-dependent TGF-β signaling is affected by smoking has not yet been investigated.

Alterations in TGF-β signaling are linked to a variety of human diseases including cancer, inflammation and tissue fibrosis (Blobe, Schiemann et al. 2000; de Caestecker, Piek et al. 2000). The disruption of TGF-β signaling occurs in several human cancers and the pathway generally possesses a tumor suppressor function (Kato, Habas et al. 2002).

However, in the later stages of carcinogenesis the tumor cells become resistant to TGF-β-induced growth arrest. Although alterations in TGF-β receptors have been implicated in the resistance to its tumor suppressor function, tumors may use various mechanisms anywhere along its primary cytoplasmic signal transducers, the Smad proteins, to circumvent the growth inhibitory effects of TGF-β (Bierie and Moses 2006; Levy and Hill 2006).

The majority of the studies performed thus far were analyzed on short-term ((Jorgensen, Dozmorov et al. 2004; Narayan, Jaiswal et al. 2004; Lemjabbar-Alaoui, Dasari et al. 2006) or long-term (Liu, Killian et al. 2010) exposure of epithelial cells to high doses of tobacco constituents. Interestingly, despite the fact that cigarette carcinogens mediate inactivation of numerous tumor suppressor genes, the relevance of cigarette smoking as to the overall prognosis of lung cancer patients remains controversial (Zhou, Heist et al. 2006; Itaya, Yamaoto et al. 2007). The present study was undertaken to ascertain if cigarette smoke induces any alterations in the Smad-dependent TGF-β signaling, which directly enhance the malignant phenotype of lung cancer cells.

Here, we describe the results of a study in which immortalized human bronchial epithelial cells, HPL1A and lung adenocarcinoma cell line A549, were chronically exposed to low doses of cigarette smoke condensate (CSC) for approximately one year. Therefore, we intended to mimic, in vitro, the long-term exposure of human lung epithelium to smoke. We found that cells exposed to smoke for a long-term makes them tumorigenic or more tumorigenic by decreasing the levels of tumor suppressor protein Smad3.

Results

Cigarette Smoke Condensate (CSC) treatment inhibits Smad-dependent TGF-β

signaling through down-regulation of Smad3

To test the effect of CSC on TGF- β signaling, we looked at the functional

complex formation between Smad2 or Smad3 and Smad4 by immunoprecipitation (IP)

assays. A549 and HPL1A cells were treated with CSC (25 μg/ml) for 4, 100 and 300 days

together with and without TGF- β for 1 hour. Lysates were subjected to IP with either

anti-Smad2 or anti-Smad3 antibody followed by immunoblotting with anti-Smad4

antibody. We observed that TGF-β-induced Smad3-Smad4 but not Smad2-Smad4

complex formation was significantly reduced in chronically CSC treated cells for 300

days, suggesting a biased role of CSC in blocking the Smad3-Smad4 complex formation

in both the cell lines. The reduced Smad3-Smad4 complex formation in the long-term

CSC treated cells (300 days) was due to reduced levels of Smad3. There was no change

observed in the levels of Smad2 or Smad4 (Fig. 16 A). We observed same results when

we performed the reverse experiment, namely IP with anti- Smad4 and immunoblotted

for Smad3 (Fig. 16 B). We observed the complex formation between Smad2,3 and

Smad4 going down even when the lysates were prepared similarly as above and were

subjected to IP with both anti -Smad2 and anti - Smad3 together (Fig. 16C).
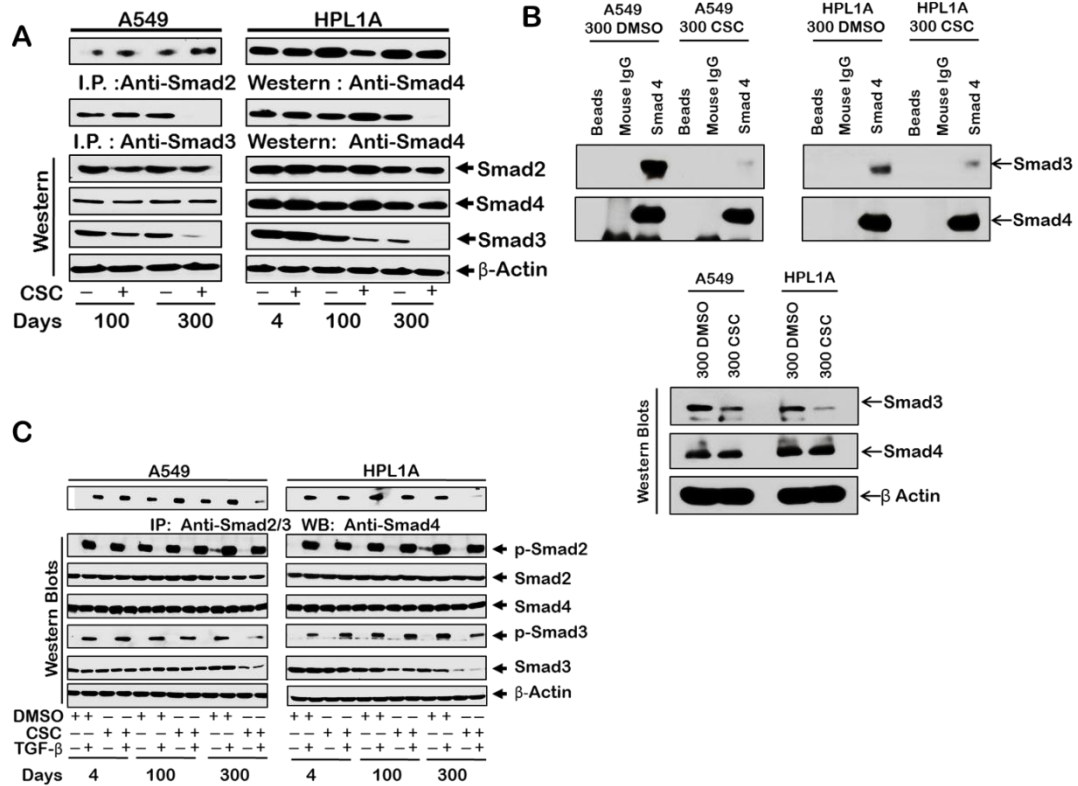
Figure16. Long-term CSC treatment abrogates Smad2/3-Smad4 complex formation by decreasing Smad3.

A. Cell lysates from the different time points of CSC treatment were serum starved overnight and treated with TGF-β1 for 1 hour and were subjected to immunoprecipitation separately with anti-Smad2 and with anti-Smad3; antibodies and the immunoprecipitates were analyzed by Immunoblot analysis with anti-Smad4 antibody (top). Cell lysates were subjected to Immunoblot analysis with anti-Smad2, anti-Smad3 and anti-Smad4 with anti-β-actin acting as a loading control.

B. Cell lysates at same condition were now subjected to immunoprecipitation with anti-Smad4 antibody and the immunoprecipitates were analyzed by immunoblot analysis with

anti-Smad3 antibody. Lower panels showing the expression Smad3 and Smad4 by Western Blot analysis.

C. Cell lysates from the same conditions were subjected to immunoprecipitation with anti-Smad2 and anti-Smad3; antibodies and the immunoprecipitates were analyzed by Immunoblot analysis with anti-Smad4 antibody (top). Cell lysates were subjected to Immunoblot analysis with anti-phospho Smad2, anti-Smad2, anti-Smad3 and anti-Smad4 with anti-β-actin acting as a loading control.
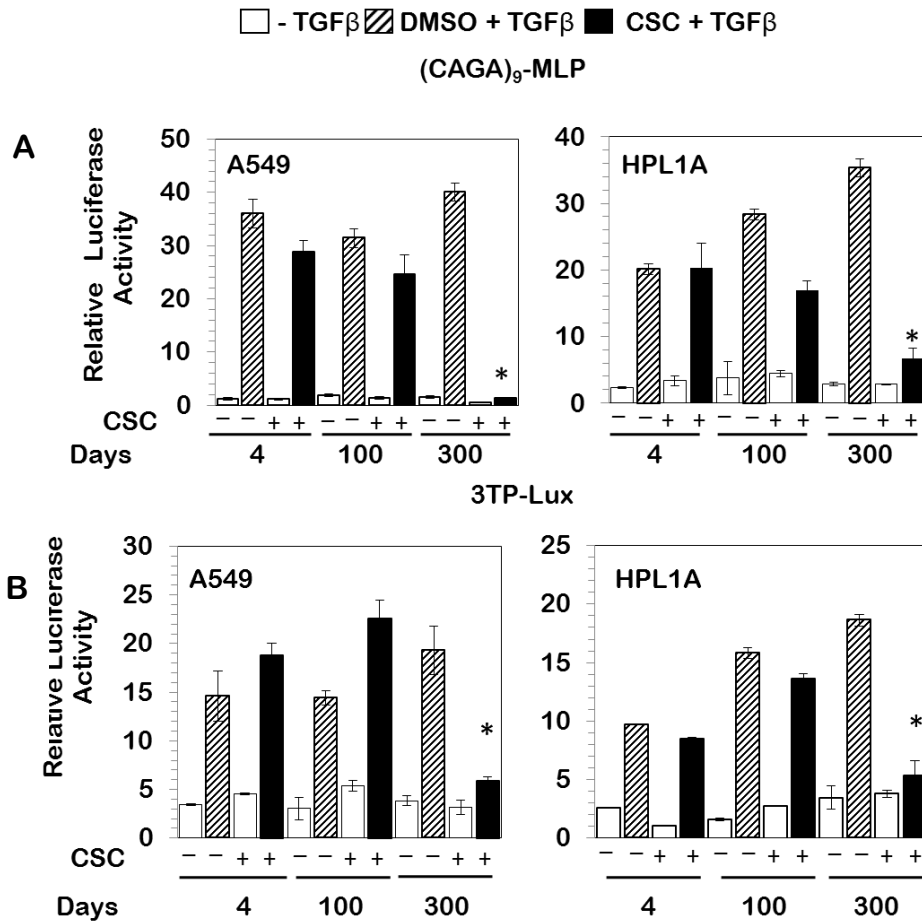
Figure17. Long-term CSC treatment decreases TGF-β signaling.

Reporter assay: CSC treated A549 and HPL1A cells were transiently transfected with CMV-β-gal and (CAGA) $_9$-MLP-Luc (A) and p3TP-Lux (B) reporter plasmids. Luciferase activity was normalized to β-gal activity, and the relative luciferase activity was expressed. Long term CSC treatment significantly reduced the TGF-β inducibility of the reporters ($_*$ p< 0.001) in both cell lines.

To test whether the inhibition of Smad complex formation affects downstream transcriptional responses mediated by TGF-β, we performed transient transfection assays using TGF-β-responsive reporters, p3TP-Lux and (CAGA)$_9$-MLP-Luc. Both the reporters (CAGA)$_9$-MLP-Luc (Fig. 17A) and p3TP-Lux (Fig. 17B) activities were reduced dramatically in 300-day CSC treated cells. Taken together these results suggest that the long-term CSC treatment decreases the Smad3-Smad4 complex formation due to a decrease in Smad3 expression and inhibits TGF-β-induced gene regulation.

CSC treatment decreases Smad3 mRNA, and the promoter activity and the expression is correlated to Histone Deacetylation

To determine whether the Smad3 mRNA levels correlate with the protein levels we performed quantitative real-time PCR in the various time points of CSC treated cells along with the controls. We observed that the Smad3 mRNA goes down in the 300-days CSC treated cells compared to the control cells (300-days DMSO) (Fig. 18A) in both the cell lines. To determine whether the reduction in Smad3 expression by CSC is at the promoter level or due to post-transcriptional changes, we transiently transfected Smad3 promoter reporter (-1878/+13) in both HPL1A and A549 cells pre-treated with CSC for 4, 100 and 300 days. Smad3 promoter activity was decreased by CSC in a time dependent manner (Fig. 18B). The Smad3 expression can go down through several epigenetic mechanisms namely histone deacetylation and DNA methylation. To test which epigenetic change, histone deacetylation or the DNA methylation, is playing a role in CSC-induced down-regulation of Smad3, 300-day CSC treated A549 and HPL1A cells were treated with either HDAC inhibitor, Sodium Butyrate TSA or the methylation inhibitor, Azacytidine. Treatment of Sodium Butyrate (Fig. 18C) suppressed CSC-mediated down-regulation of Smad3, whereas Azacytidine had no significant effect (data not shown). When we preformed ChIP assay using Sp1, a known transcription repressor shown to be involved in Smad3 transcription (Lee, Elmer et al. 2004), we observed an increase in occupancy of Sp1 on the Smad3 promoter in the 300-days CSC treated cells and it vanishes due to Sodium Butyrate treatment (Fig. 18D). These results suggest that long-term CSC treatment decreases Smad3 mRNA and Smad3 promoter activity and histone deacetylation may play a role in reducing the Smad3 expression.
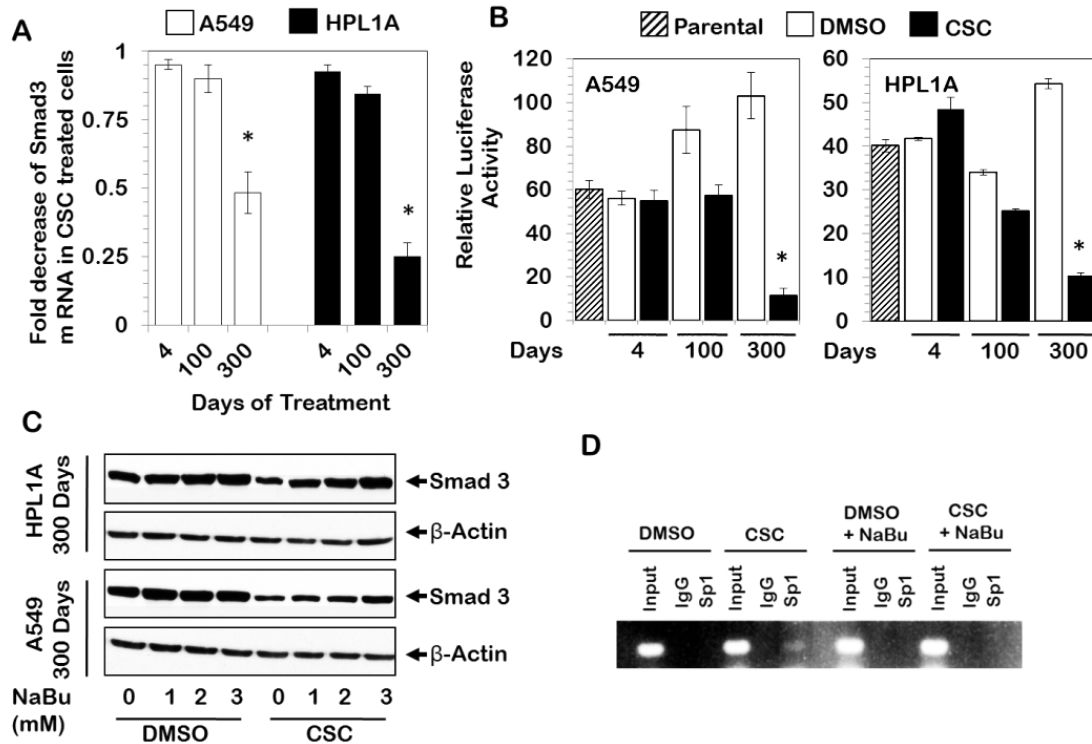
Figure 18. Long-term CSC treatment decreases expression of Smad3 at both protein and mRNA level and the decrease is correlated to histone deacetylation

A. Quantitative RT-PCR: Quantitative RT-PCR was used to determine the fold decrease of *Smad3* mRNA, normalized to internal control *GAPDH*. The fold decrease was calculated by normalizing to the level of *Smad3* mRNA in the corresponding DMSO treated samples. Long term CSC treatment significantly reduced the *Smad3* mRNA compared to controls ($*$ p< 0.001).

B. Reporter assay: 4, 100 and 300-day CSC treated cells were transiently transfected *Smad3* promoter reporter in both HPL1A and A549 cells. Luciferase activity was normalized to β-gal activity, and the relative luciferase activity was expressed. Long

term CSC treatment significantly decreased the Smad3 promoter activity ($*$ p< 0.001) in both cell lines

C. 300-day treated A549 and HPL1A cell lines were treated with either HDAC inhibitor or Sodium Butyrate (1, 2, 3mM) and the protein levels were checked by immunoblot analysis using anti-Smad3 antibody. An equal amount of protein loading was verified by immunoblot analysis with anti-β-actin monoclonal antibody.

D. ChIP assay: ChIP assay was performed on 300-day CSC and DMSO treated samples to determine the occupancy of transcriptional repressor Sp1 on the Smad3 promoter. The sixth lane from the left shows the occupancy of Sp1 on the Smad3 promoter due to 300-day CSC treatment. The occupancy of Sp1 on Smad3 promoter vanishes due to HDAC treatment.

CSC treatment inhibits TGF-β-induced growth suppression and attenuates apoptosis by up-regulation of Bcl-2

To determine if long-term CSC treatment has any effect on cell viability, we checked for cell viability by MTT assay in both A549 and HPL1A cells. We also tested whether the decrease in the Smad-dependent TGF-β signaling has any effect on TGF-β-induced growth suppression. We found that the long-term CSC treated cells grew faster than the corresponding DMSO treated cells. We also observed that the TGF-β-induced growth suppression was diminished in the long term CSC treated cells (Fig. 19A). These data suggest that long-term CSC treatment increases cell growth and decreases TGF-β-induced growth suppression. The increase in cell viability could be due to an increase in cell proliferation or decrease in apoptosis. We tested the cell proliferation by Thymidine incorporation assay. We did not see a significant change in cell proliferation by long-term CSC treatment (data not shown). To determine whether CSC treatment has any effect on apoptosis, we performed quantitative ELISA assay, which detects the cytoplasmic histone associated DNA fragments (mono and oligonucleosomes) after cell death. We found that long-term CSC treatment (300-days) of both A549 and HPL1A cells led to less apoptosis in CSC treated cells compared to their corresponding DMSO controls (Fig. 19B), suggesting a role of long-term smoking in inhibiting apoptosis. This was confirmed by a qualitative DNA laddering assay in which the 300-day DMSO treated cells showed much more DNA laddering than the corresponding CSC treated cells (Fig. 19C). To understand the mechanism of decreased apoptosis, we assayed a number of important apoptotic regulators including Bcl-2, Bcl-xl, Bcl-w, and Bax in CSC treated A549 and HPL1A cells. Immunoblot analyses indicated that the level of anti-apoptotic Bcl-2 was up-
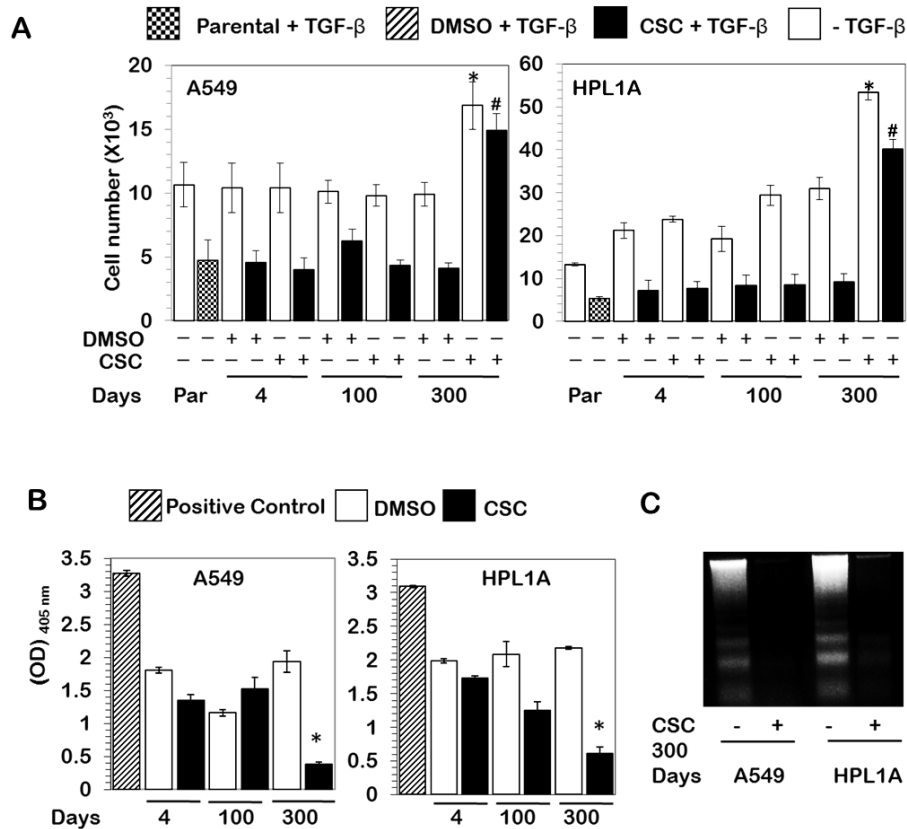
Figure 19. Long-term CSC treatment inhibits TGF-β-induced growth suppression and apoptosis.

A. Cell viability of CSC treated cells in presence and absence of TGF-β determined by MTT assay. Long term CSC treatment significantly increased the growth in absence of TGF-β(* p< 0.001), and in presence of TGF-β(# p <0.001) in both cell lines.

B. Quantitative cell death by ELISA in the various time points of CSC treated cells. Long term CSC treatment decreases the apoptosis significantly (* p< 0.001) in both cell lines.

C. DNA laddering of A549, HPL1A 300-day CSC and DMSO treated cells cultured in serum-free medium for 72 hours.

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regulated in cell extracts prepared from the 300-day CSC treated cells. No detectable

change of other members of the Bcl-2 family was observed (Fig. 20A), and the pro-

apoptotic members namely Bax remains unchanged. To validate whether Bcl-2 is the

cause of increase in apoptosis, we performed Bcl-2-siRNA knockdown in the 300-day

CSC treated A549 and HPL1A cells. We assayed for apoptosis 72 hours after transfection

of 20 nM of each Bcl-2 siRNA and scrambled control. We observed that apoptosis goes

up (Fig. 20C) upon Bcl-2 knockdown. Simultaneously, we also checked the protein

expression of Bcl-2 to confirm whether Bcl-2 levels are decreasing (Fig. 20B). Since NF-

kB is known to up-regulate Bcl-2 and to rule out the possibility that NF-kB is responsible

for up-regulating Bcl-2, we checked the expression of NF-kB in the long-term CSC

treated cells. We do not see an increase of NF-kB (p65) levels (Fig. 20D) due to long-

term CSC treatment, suggesting the up-regulation of Bcl-2 is not dependent on NF-kB in

our model system. These results suggest that CSC treatment increases cell viability by

decreasing apoptosis through up-regulation of anti-apoptotic Bcl-2.
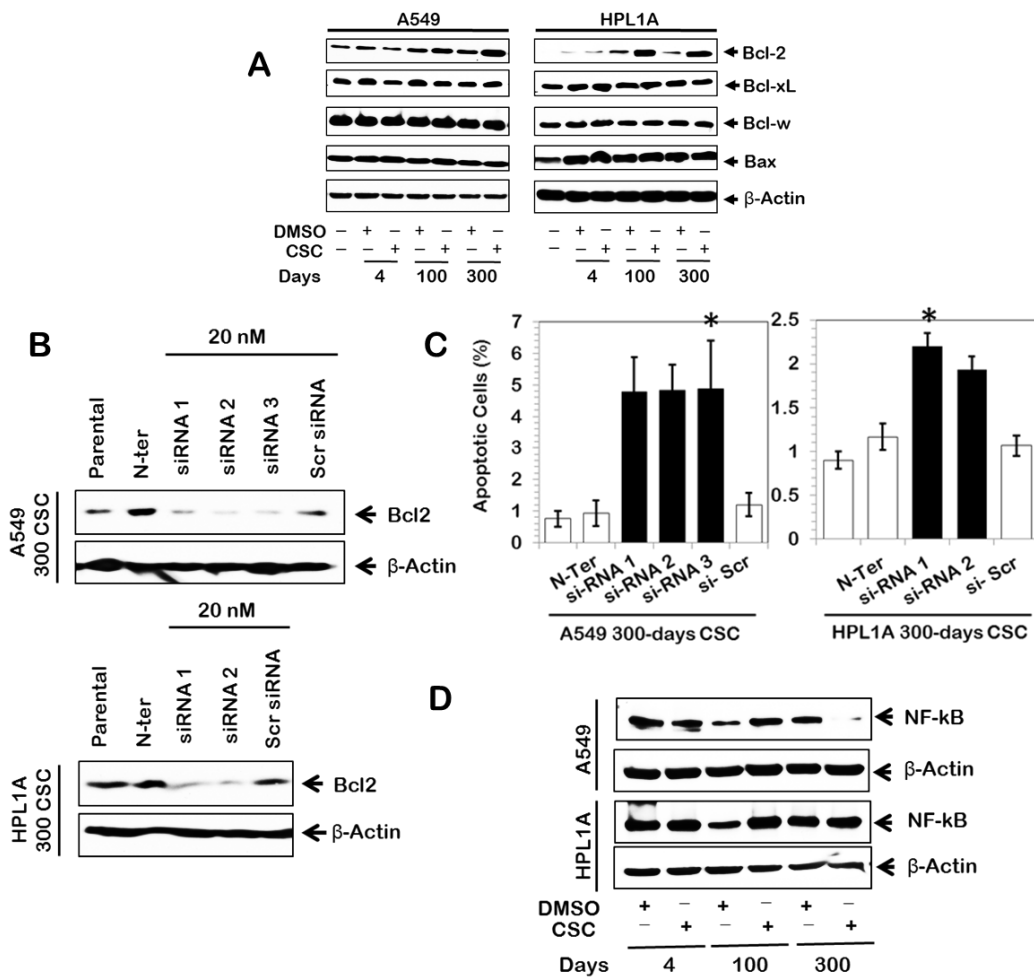
Figure 20. Long term CSC treatment decreases apoptosis by increasing Bcl2 independent of NF-kB.

A. Cell lysates from various time points of CSC treatment were analyzed by immunoblot analysis with pro-apoptotic anti-Bcl-2, anti-Bcl-xl, anti-Bcl-w, and anti-apoptotic Bax antibody. An equal amount of protein loading was verified by immunoblot analysis with anti-β-actin antibody.

B and C. 300-day CSC treated cells were transiently transfected with (20nM) of each Bcl-2 siRNA and assayed for apoptosis by FACS analysis. (B) Western blot showing the knockdown of Bcl2 in the 300-day CSC treated cells. (C) The percentages of cells

undergoing apoptosis are represented in the graph. Knocking down Bcl-2 increases the apoptosis in long term CSC treated cells significantly (* p<0.001) in both cell lines.

D. Cell lysates from various time points of CSC treatment in both A549 and HPL1A cells were analyzed by immunoblot analysis with NF-κB. An equal amount of protein loading was verified by immunoblot analysis with anti-β-actin antibody.

Withdrawal of CSC treatment restores TGF-β signaling and apoptosis

After smoking cessation, the cumulative death risk from lung cancer decreases. Peto et al. showed that, the earlier cigarette smoking is stopped the greater the decline in lung cancer mortality (Peto, Darby et al. 2000). There is a pressing need to clarify the role of smoking cessation in the care of lung cancer patients. To mimic the conditions of smoking cessation, we withdrew cells from the long-term (300-day) CSC treatment and tested the effects on TGF-β signaling. We observed that Smad3-Smad4 complex formation goes up with the withdrawal of CSC treatment in the 300-day CSC treated samples. Thus, the Smad-dependent TGF-β signaling is restored together with Smad3 expression (Fig. 21A). To test whether this restoration in TGF-β signaling translates to biological outcomes namely a decrease in cell viability, MTT assay was performed using both A549 and HPL1A cells after withdrawing CSC treatment. We saw that cell viability decreases and TGF-β mediated growth inhibition increases with withdrawal of CSC treatment (Fig. 21B). To test whether withdrawal of CSC treatment has any effect on apoptosis of cells, we performed cell death with the ELISA assay. Withdrawal of CSC treatment brings back apoptosis (Fig. 21C). These results suggest that the effect of CSC

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treatment is reversible and that the withdrawal of CSC treatment restores TGF-β

signaling, apoptosis and reduce cell viability.
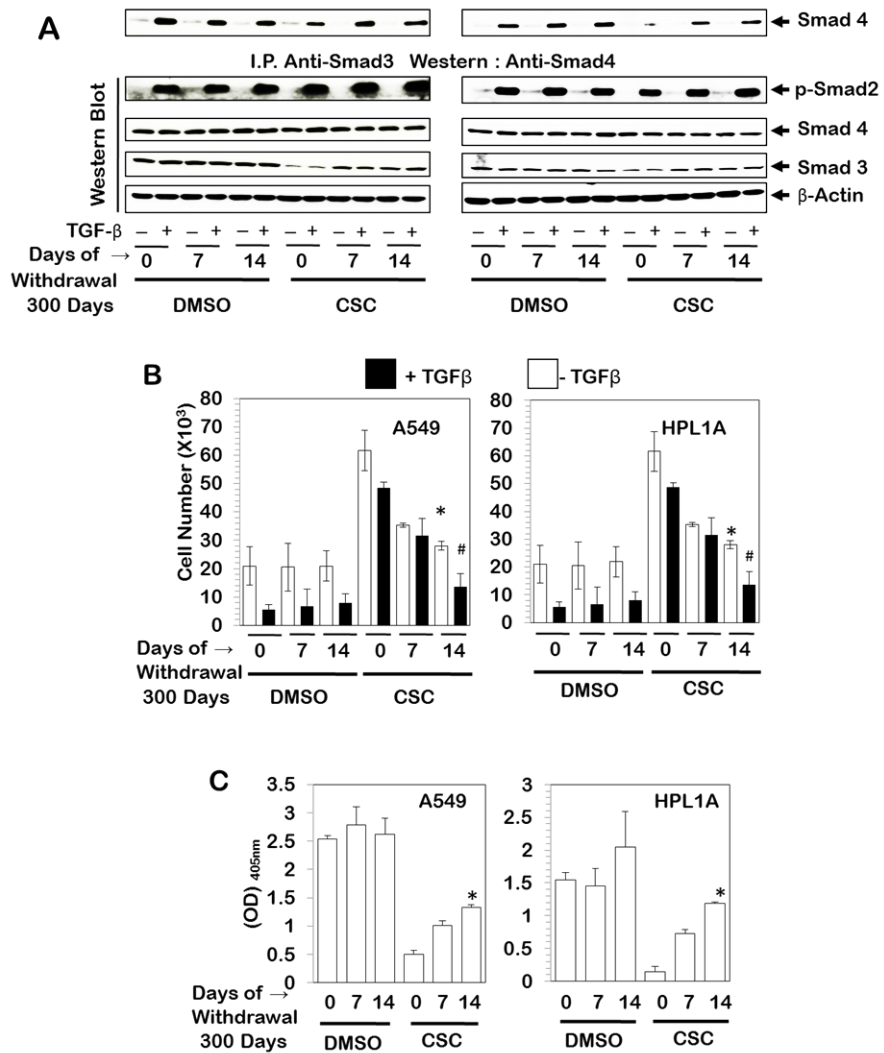
Figure21. Withdrawal of CSC treatment restores Smad-dependent TGF-β Signaling

and apoptosis.

A. Cell lysates from 7 and 14 day CSC withdrawal cells after treatment with CSC for 300 days were treated with TGF-β for 1 hour were immunoprecipitated with anti-Smad3 and immunoblotted for anti-Smad4.

B. Cell viability of 300-day CSC and 7-, 14- day CSC withdrawal cells in presence and absence of TGF-β determined by MTT assay. Withdrawal of CSC treatment significantly decreased the growth in absence of TGF-β(* $p < 0.001$), and in presence of TGF-β(# $p < 0.001$) in both cell lines.

C. Quantitative cell death by ELISA in the 300-day CSC treated and 7, 14 days CSC withdrawal cells. Withdrawal of CSC treatment significantly increases the apoptosis (* $p < 0.001$) in both cell lines.

Stable expression of Smad3 restores TGF-β signaling, apoptosis and reduces cell viability in long-term CSC treated cells

CSC contains thousands of compounds comprising five known human carcinogens and many toxic agents. These may affect many different pathways ultimately leading to increased cell viability and altered apoptosis. To verify that the increase in cell viability is due to the decrease in Smad3 expression we performed rescue experiments, where we overexpressed Smad3 in 300-day CSC treated cells (Fig. 22A). Stable overexpression of Smad3 in both the 300-day CSC treated cell lines, HPL1A and A549, brought TGF-β signaling back as demonstrated by the luciferase assays with TGF-β responsive reporter elements both p-3TP-Lux and (CAGA)9-MLP (Fig. 22 B and 22C). Similarly, the biological outcomes of the long-term CSC treatment increased cell viability and decreased apoptosis were also rescued by overexpression of Smad3. We observed that clones of Smad3 overexpressing in both 300-day CSC treated HPL1A and A549 cell lines underwent more apoptosis compared to the 300-day CSC treated HPL1A and A549 cell lines and vector controls when we performed cell death by ELISA (Fig. 23 A and B, left panels). We observed that the same clones were less viable compared to the 300-day CSC treated HPL1A and A549 cell lines and the vector controls (Fig. 23A and B, right panels). These combined results suggest that the increase in cell viability by decreasing apoptosis is imparted via long-term CSC treatment, partly through down-regulation of Smad3.

A

Smad3

β-Actin

1 2 3 12 22
V   Smad3

A549 300 CSC

Smad3

β-Actin

V 1 6 5 2
    Smad3

HPL1A 300 CSC

B

(CAGA)₉-MLP

p-3TP-Lux

A549 300 days CSC Treated Cells

C

(CAGA)₉-MLP

p-3TP-Lux

HPL1A 300 days CSC Treated Cells
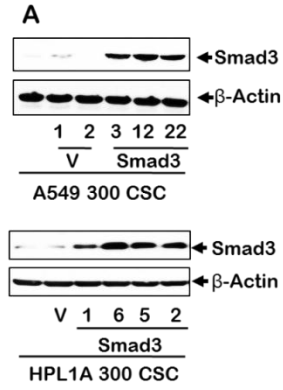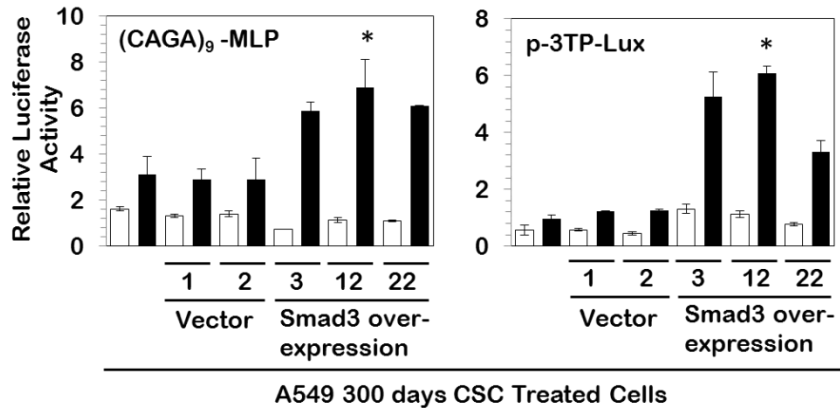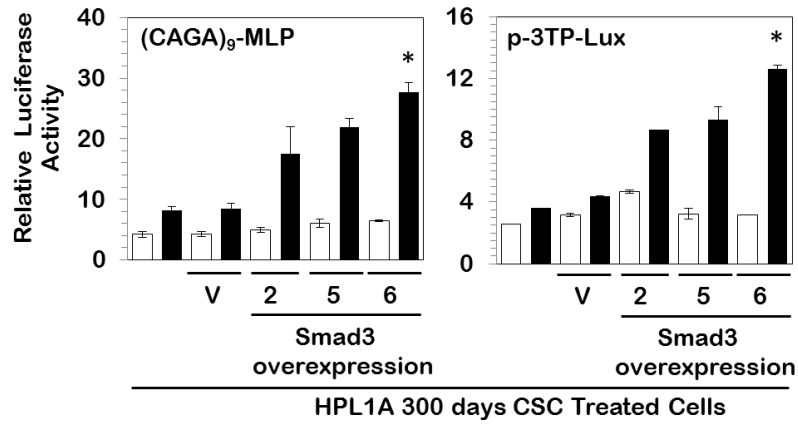
Figure 22. Stable expression of Smad3 restores TGF-β signaling.

A. Cell lysates from parental, vector control, and three stable Smad3 overexpression clones were analyzed for overexpression of exogenous Smad3 by immunoblotting with anti-Smad3 antibody (left). β-Actin was used as a loading control for an equal amount of proteins.

B,C. Luciferase assays were performed 300-day CSC treated A549 (B) and HPL1A (C) cells stably transfected with Smad3 expression plasmid. Cells along with vector controls were transiently transfected with CMV-β gal, and p3TP-Lux-Luc or $(CAGA)_9$-MLP-Luc reporter plasmids. Transfected cells were incubated in 0.2% FBS containing medium with 5 ng/ml TGF-β1 for 22 hours. Cell lysates were used to measure both luciferase and β-gal activities and the normalized luciferase activity was presented.
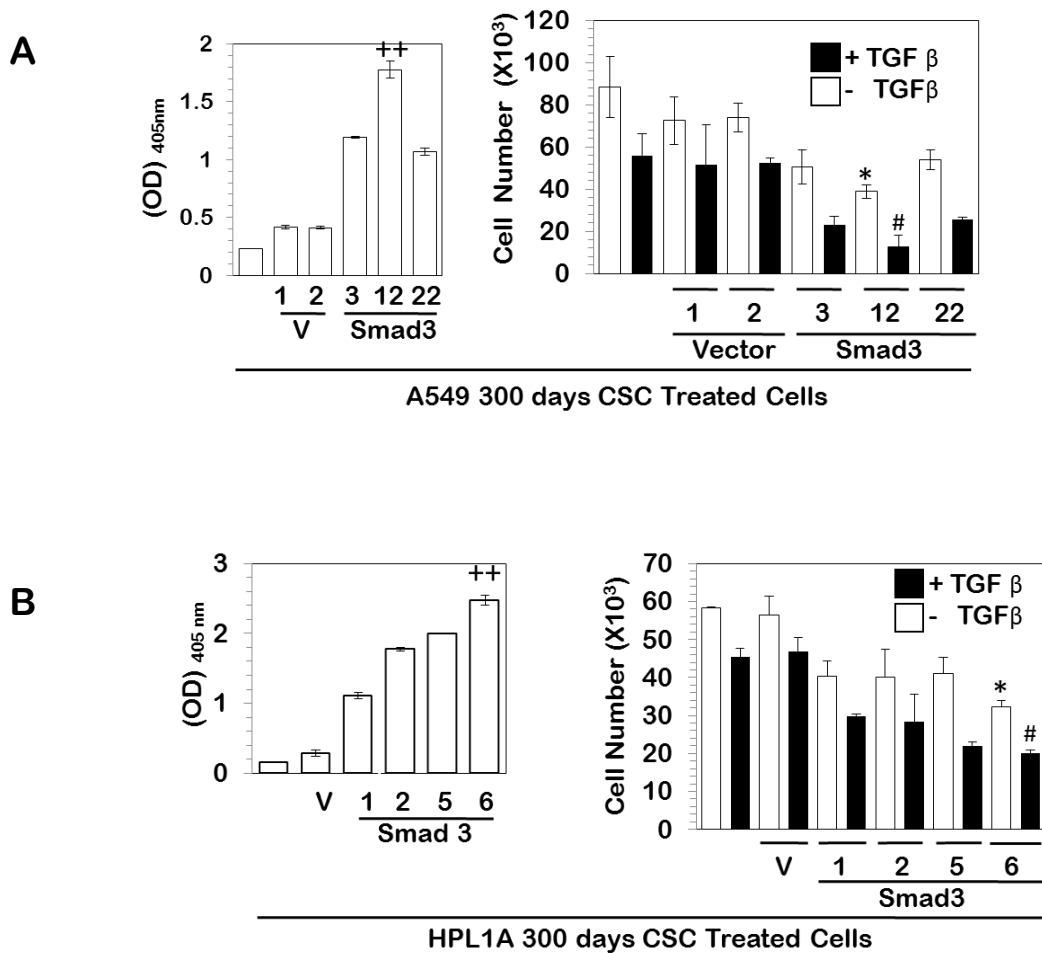
Figure 23. Stable expression of Smad3 reduces cell viability and induces apoptosis in 300-day CSC treated cells.

Cell viability by MTT assay and quantitative cell death by ELISA. Cells from parental, vector control and three stable Smad3 clones were tested for cell viability both in presence and absence of TGF-β (5ng/ml)(right panels) and apoptosis by ELISA (left panels) in 300-day A549 (A) and in HPL1A (B). Overexpression of Smad3 significantly increased apoptosis (++ p<0.001),   decreased growth in absence of TGF-β (* p< 0.001), decrease in growth in presence of TGF-β (# p <0.001) in both cell lines.

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Long-term CSC treatment enhances tumorigenicity of the cell both in vitro and in vivo partly by down-regulating Smad3

Since the increase in cell viability and decrease in apoptosis by chronic exposure of cells to CSC are reminiscent of the phenotypes characteristic of oncogenically transformed cells, we next examined whether CSC treated cells were capable of anchorage independent growth by culturing them in soft agar. We performed soft agar assay using CSC treated A549 cells along with corresponding DMSO controls. The size and the number of colonies in the 300-day CSC-exposed cells were increased significantly when compared to the corresponding DMSO treated cells (Fig. 24A). We also observed that 300-day CSC treated A549 cells formed tumors bigger and faster than the 300-day DMSO and parental A549 cells (Fig. 24B). Taken together, these data suggest chronic CSC treatment not only enhances oncogenic transformation as elucidated by the soft agar assay but also enhances the tumorigenicity in the A549 cells.

To verify that the increase in anchorage independent growth and tumorigenicity is due to the decrease in Smad3 expression we performed rescue experiments, where we overexpressed Smad3 in 300-day CSC treated cells. We performed soft agar assay using Smad3 overexpression clones and the corresponding vector controls. The number and the size of colonies were fewer in the Smad3 overexpression clones when compared to the vector controls and 300-day CSC treated cells (Fig. 25A). We also observed that the tumors formed in nude mice by Smad3 overexpression clones were smaller and slower than the vector controls and 300-day CSC treated cells (Fig. 25B). Taken together, these data suggest that long-term CSC treatment partly enhances the tumorigenicity of the

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cells, and thus, partly contributes to smoking-induced lung cancer development by reducing the levels of Smad3.
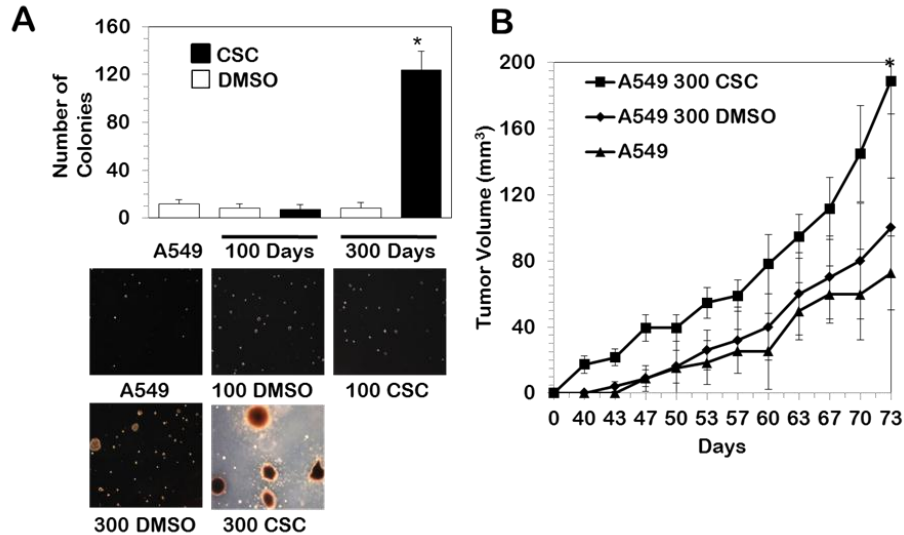
Figure 24. Long-term CSC treatment increases anchorage-independent growth and increases tumorigenicity.

A. Growth in soft agar of A549 and various time points of CSC exposed cells. Pictures of colonies grown in soft agarose (bottom). Quantification of the colonies is shown on the top. Columns, average of three independent values determined from three plates (top); bars, SD. Long term CSC treatment significantly increased the number of colonies (* p< 0.001).

B. Cells from parental, 300-day CSC and 300-day DMSO were injected s.c. in athymic nude mice. The animals were monitored for tumor formation for a total of 12 weeks. Tumors were measured externally and volume was determined. Points, mean volume of tumors from four mice in each group; bars, SD. Long term CSC treatment significantly increased the tumor volume (* p< 0.001) and produced tumors faster (# p<0.01).

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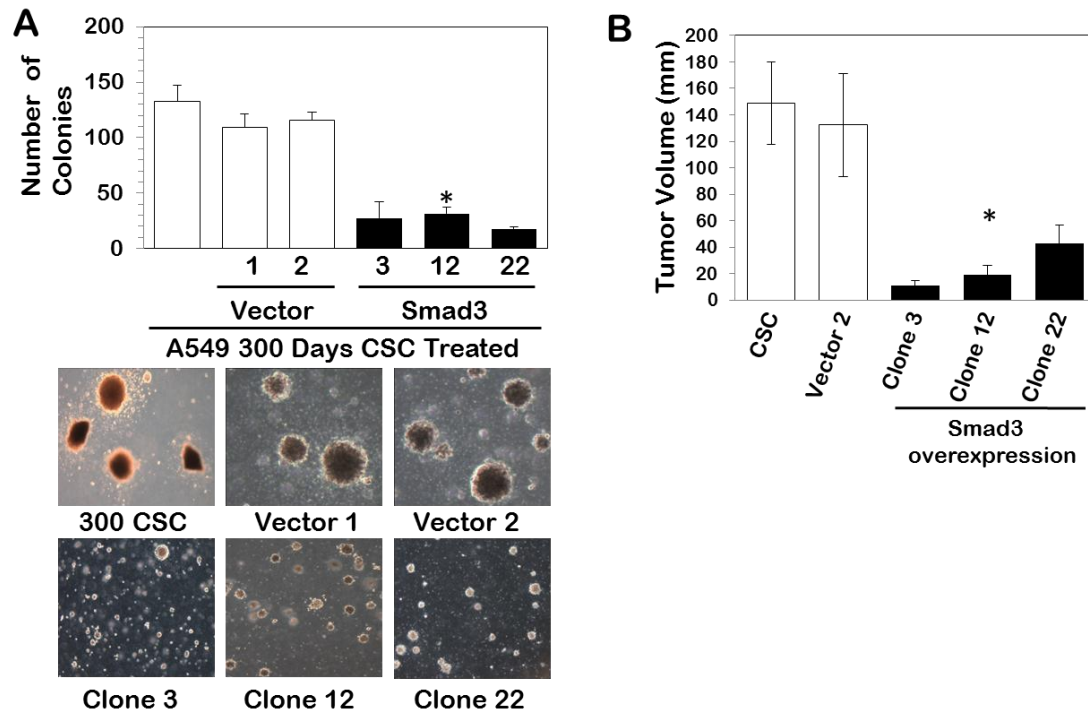
Figure 25. Long-term CSC treatment increases anchorage-independent growth and increases tumorigenicity partly by down regulation of Smad3.

A. Growth in soft agar assay of Smad3 overexpressing 300-day CSC treated A549 and vector control cells. Pictures of colonies grown in soft agarose (bottom). Quantification of colonies is shown on the top. Columns, average of three independent values determined from three plates (top); bars, SD. Overexpression of Smad3 decreased the number of colonies significantly (* $p < 0.001$).

B. Cells from A549 300-day CSC and Smad3 overexpressed A549 300-day CSC treated, along with vector control were injected s.c. in athymic nude mice and monitored for tumor formation for a total of 12 weeks. Tumors were measured externally and volume was determined. Points, mean volume of tumors from four mice in each group; bars, SE.

Overexpression of Smad3 in long term CSC treated cells decreased the tumor volume significantly (* p< 0.001).

Smad3 status and its correlation with patient survival and smoking

To elucidate the clinical relevance of our in vitro results that Smad3 expression goes down due to long-term CSC treatment, we performed immunohistochemical staining of tissue micro array (TMA) using the anti-Smad3 antibody. We observed that the staining of Smad3 in cancer cells was mostly nuclear (Fig 26). We also observed strong staining of Smad3 in the stroma and macrophages. The frequency of positive staining (>0) was more in the case of adenocarcinoma compared to the other subcategories of lung cancer namely small cell lung cancer, squamous cell lung cancer and carcinoma. We plotted the intensity scores of smokers and non-smokers and we observed that the Smad3 intensity scores was less in smokers than nonsmokers (Fig. 27A). When the staining intensities were correlated with survival status of the corresponding patients, the tumor-related survival was much better for the tumors with a high intensity (>2) of Smad3 staining compared to those tumors with a low intensity (<2) (Fig. 27B). To further strengthen our observations, we looked into a public database (Shedden, Taylor et al. 2008) to check the Smad3 mRNA expression in smokers, never-smokers and former-smokers in lung cancer patients. We found that Smad3 is expressed in lower levels in smokers compared to never-smokers (p=0.0396); Smad3 expression is lower in former-smokers compared to never-smokers (p=0.0254) (Fig. 28). Taken together these data suggests that Smad3 expression is lower in lung cancer patients who are current smokers and former-smokers compared to never-smokers.

Intensity: +3
Smad3 stained nuclei: 80%

Intensity: +1
Smad3 stained nuclei: 20%

Former Smoker

Current Smoker

Squamous Cell Carcinoma

Intensity: +2
Smad3 stained nuclei: 60%

Intensity: 0
Smad3 stained nuclei: 0%

Never Smoker

Current Smoker

Adenocarcinoma
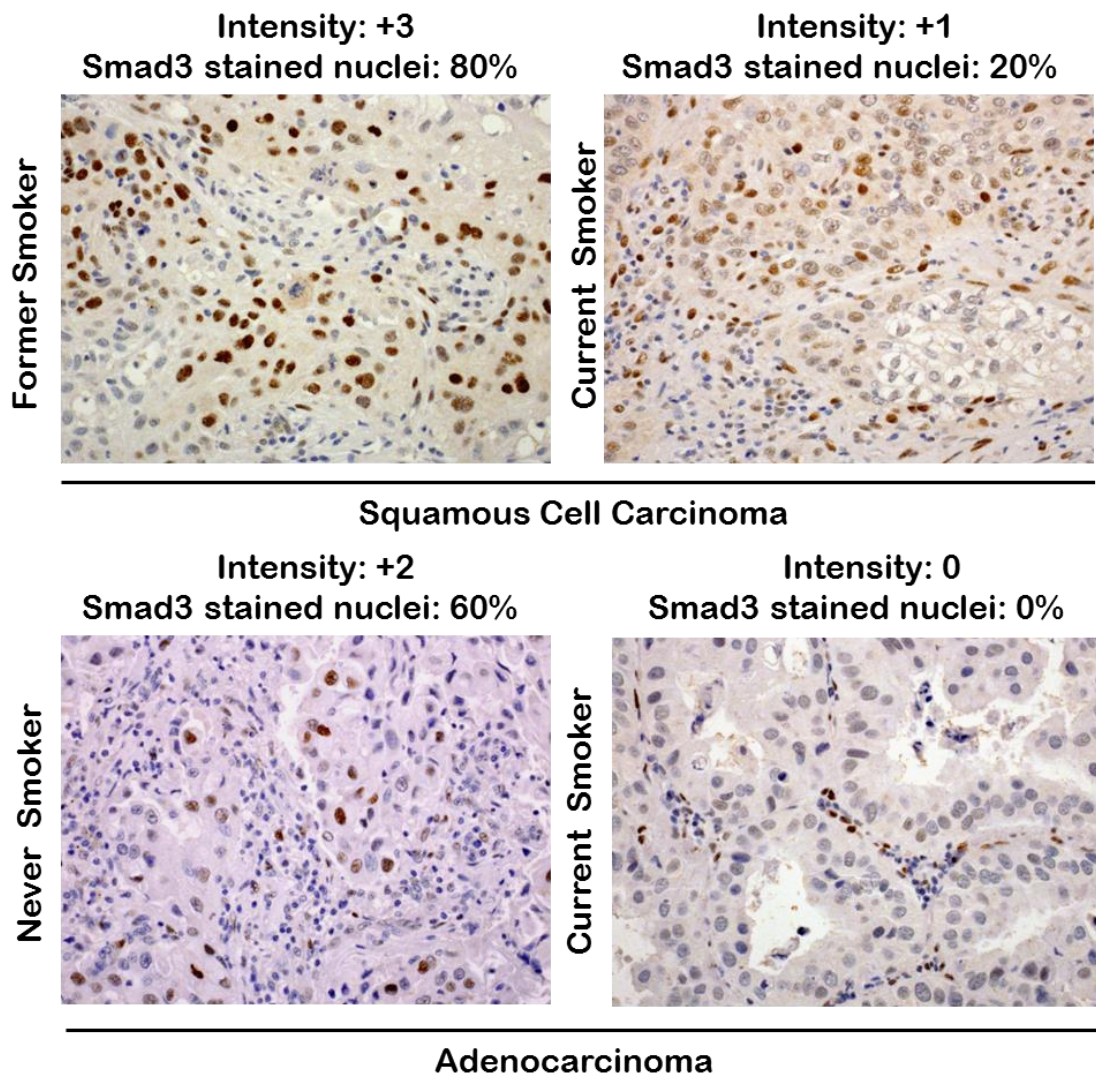
Figure 26. Smad3 immunohistochemistry and tumor-related survival in lung cancer patients.

A. Representative images of immunohistochemistry staining for Smad3 in lung cancer patients (200X Magnification).
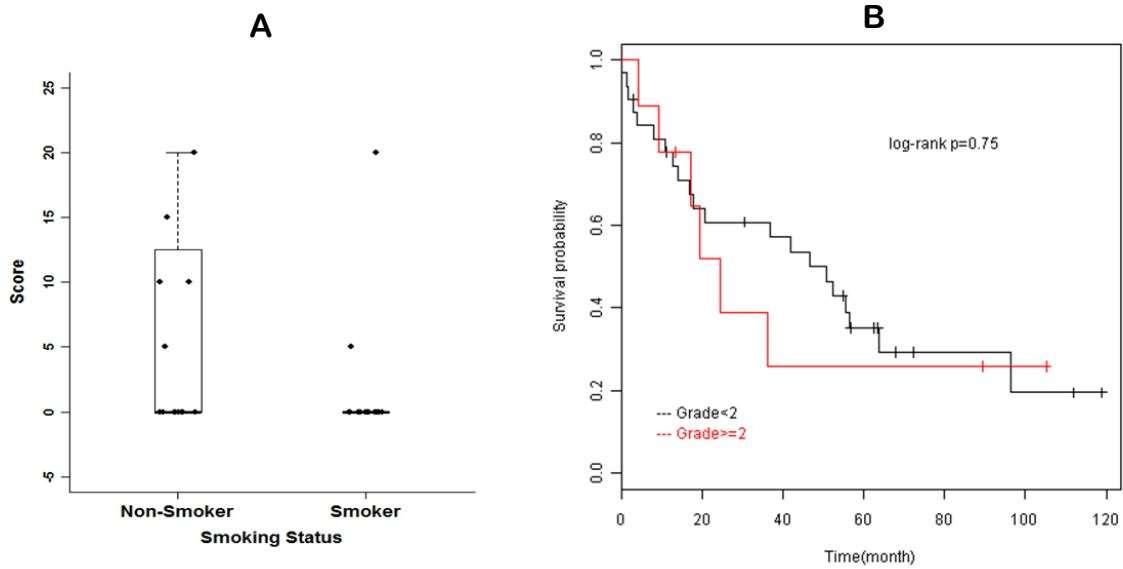
Figure 27. Quantitation of Smad3 immunohistochemistry and tumor-related survival in lung cancer patients.

A. Box plot for Correlating Smad3 and smoking: Smokers (including current smokers and former-smokers) and never-smokers were categorized and Smad3 intensity scores (Intensity X Percentage of Cells) were plotted and the box plot was generated. * p= 0.15 for intensity scores of Smad3 by t-test.

B. Kaplan-Meier Curve for tumor related survival status based on Smad3 status. Patients with high Smad3 tumors (Intensity >=2) were compared to patients with moderate or low expressions of Smad3 (Intensity 0, 1 and 2). * p= 0.75 by log rank test.
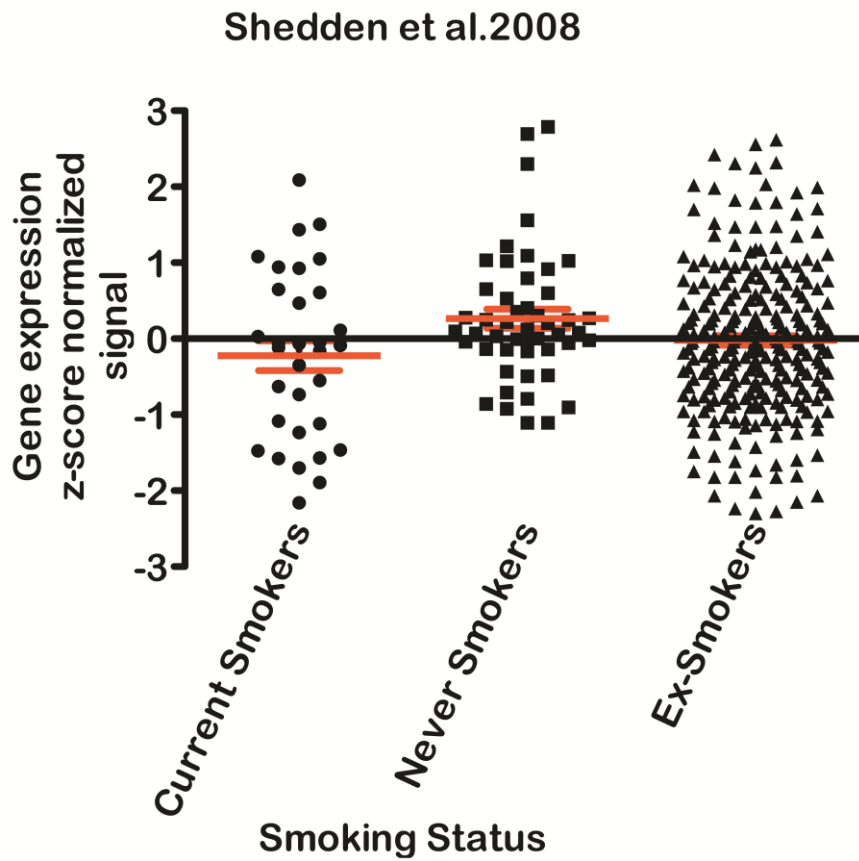
Figure 27. Smad3 expression according to the smoking status.

Scatter plot for *Smad3* from Shedden et al. 2008: Gene expression normalized z scores of *Smad3* from lung cancer patients are plotted categorized by smoking status into Current smokers, Never-Smokers and Former-smokers. p values were calculated for the pairs shown by t-test.

Discussion

Lung carcinogenesis involves the accumulation of genetic and epigenetic changes that accumulate over a long course due to chronic smoking or other genetic susceptibility factors (Herbst, Heymach et al. 2008).One of the putative problems in all previous studies is that the cells were treated with tobacco constituents for a maximum of 10 days. As a result, short-term exposure to cigarette smoke cannot be a true representation of lung cancer, which mainly occurs in long-term smokers.

In this study, we have shown that long-term exposure to CSC can damage lung epithelial cells, inducing hyperplasic growth and carcinogenesis. Our results reveal that SV40-immortalized bronchial epithelial HPL1A cells and lung adenocarcinoma cell line A549 surviving 300 days of repeated CSC exposure are endowed with some phenotypic changes that are characteristic of oncogenic transformation: alteration in growth kinetics, decrease in the tumor suppressing effects of TGF-β, decrease in apoptosis due to an increase in oncogenic anti-apoptotic Bcl-2. In addition, the A549 cell line with 300 days of CSC treatment has increased anchorage independent growth, and more importantly, the ability to produce tumors in nude mice (Fig.24). Therefore, this approach of treating cells long-term with CSC at low concentration, offers a new tool to study the biology of lung cancer in long-term smokers. One putative issue in culturing the HPL1A cells could be culturing these cells in media supplemented with 10% FBS instead of 1% FBS that was used for establishing the cell line (Masuda, Kondo et al. 1997)). We have used 10% FBS containing medium to culture these cells for several reasons, 1) there are no changes in TGF-ß signaling when these cells are grown in 10% serum (Yanagisawa, Osada et al.

1998) , 2) 10% FBS containing medium was used to grow HPL1A cells before (Sato, Yazawa et al. 2006), 3) HPL1A cells cannot survive long-term (300 days) in culture without serum and 4) TGF-ß-induced transcriptional regulation and growth inhibition (Fig. 17, A, B right panels and Fig. 19A) were not affected and these cells did not become tumorigenic when grown in 10% serum containing medium (data not shown).

In the present study, an in vitro model system was used to gain insight regarding how Smad dependent TGF-β signaling alterations potentially contribute to the initiation and early progression of smoking-induced lung cancers. We show that long-term CSC treatment results in the reduced expression of Smad3 (Fig. 16). We have observed decreased Smad3-Smad4 complex formation and reduced TGF-β-induced transcription from p3TP-Lux and (CAGA)$_9$-MLP-Luc reporters (Fig. 17 A and B). This suggests that reduced expression of Smad3 inhibited TGF-β signaling. Functionally, Smad3 reductions lead to an increase in cell viability. The increase in cell viability was due to decreased apoptosis. This decrease in apoptosis was due to up-regulation in anti-apoptotic Bcl-2, as seen in the chemically induced model of HCC (Yang, Zhang et al. 2006) (Fig. 20A).

We also observed that the long-term CSC treatment increases the tumorigenicity of the cells (Fig. 24 A and B). Cigarette smoke contains about 4800 chemical agents including more than 60 known human carcinogens (Hoffmann, Hoffmann et al. 2001). The increase in tumorigenicity could be due to the activation of oncogenic pathways and/or inactivation of the tumor suppressive signaling cascade. In an attempt to elucidate the role of reduced expression of Smad3 in response to CSC treatment, we have observed that restoration of Smad3 expression in long-term CSC treated cells reduces tumorigenicity (Fig. 25 A and B). These results suggest that smoking-induced lung

cancer progression is partly due to the reduction in Smad3 expression and attenuated TGF-β tumor suppressor function. This is in agreement with the previous data that ectopic expression of Smad3 reduces the susceptibility to hepatocellular carcinoma (HCC) in a chemically induced murine model, consolidating the evidence for the tumor suppressor function of Smad3 (Yang, Zhang et al. 2006).

The failure of the long-term CSC treated HPL1A cells to grow in soft agar or orthotopically in nude mice (data not shown) indicates that additional alterations are required to facilitate tumor formation. The ability to confer a complete malignant phenotype would likely require additional epigenetic or genetic changes. Only A549 cells bear a K-ras codon 12 mutation (Takezawa, Okamoto et al. 2009). We observed enhanced tumorigenicity in long-term CSC treated A549 cells both in vitro and in vivo (Fig. 24 A and B). Therefore, one plausible explanation is that the K-ras mutation, which is present only in A549 cells, is contributing to the tumorigenicity in vitro and in vivo.

We next validated the conclusions drawn from the in vitro model in real life smokers using lung tumors of patients and public database, which provides the largest available set of microarray data with extensive pathologic and clinical annotation for lung adenocarcinomas (supporting material). In our TMA staining we saw a trend that the expression of Smad3 is less in smokers compared to non-smokers. Similarly, in public database we found that Smad3 expression is less in current smokers compared to the never-smokers and former-smokers. Thus, we showed the relevance of Smad3 expression, which is down-regulated in our in vitro model, to lung adenocarcinoma gene expression pattern found in smokers. To our knowledge, this is first time; Smad3 is correlated with the smoking status of lung cancer patients.

Our results reveal, for the first time, that a smoking-mediated decrease in Smad3 expression plays a key role in the induction of lung cancer by increasing cell viability and decreasing apoptosis. This is in accordance with the expression of Smad3 from clinical patient samples which correlates with the results obtained from our in vitro system. Since the decrease in Smad3 expression is reversible, it would suggest that it happens in the early phases of smoking-induced lung cancer. Hence, it can be potentially used as a biomarker for smoking-induced lung cancer.

CHAPTER IV

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide (Siegel, Naishadham et al. 2012). Cigarette smoking is a major risk factor in the development of non-small cell lung cancer (NSCLC), which accounts for 80% of all lung cancers (Minna, Fong et al. 2002; Sekido, Fong et al. 2003). Current standard therapies include surgical resection, platinum-based doublet chemotherapy, and radiation therapy alone or in combination. Unfortunately, these therapies rarely cure the disease, and the overall 5-year survival rate is still only 15% (Siegel, Naishadham et al. 2012). NSCLC is characterized by its poor prognosis and resistance to the apoptotic activity of anti-neoplastic drugs both in vivo and in vitro (Bepler 2003; Katakami, Sugiura et al. 2004).

cis-Diamminedichloroplatinum (II) Cisplatin, carboplatin and oxaliplatin are platinum-based drugs that are widely used in cancer chemotherapy. These agents are commonly administered in various tumor types including ovarian, cervical, head and neck, and non-small-cell lung cancer (Kelland 2007). Due to the low chloride ion concentration in the cell, cisplatin undergoes aquation to form (Pt (NH3)2Cl (OH2)) + and (Pt (NH3)2(OH2)2)2+ once inside the cell. Although many cellular components interact with cisplatin, DNA is the primary biological target of the drug (Jamieson and Lippard 1999). The platinum atom of cisplatin forms covalent bonds to the N7 positions of purine bases to afford primarily 1, 2- or 1, 3-intrastrand crosslinks and a lower number of interstrand crosslinks. The final cellular outcome of DNA adduct formation is generally apoptotic cell death. Apoptosis is thought to occur through halting of cellular

processes such as replication and transcription, leading to prolonged G2 phase cell cycle arrest and deregulation of signal transduction pathways involved in normal homeostasis of the cell, response to stress (Siddik 2003; Stewart 2007). Many factors contribute to the cellular mechanisms of resistance to platinum-based chemotherapeutics and contribute to a severe limitation in their use in clinical practice. One important mechanism acting downstream of the initial reaction of cisplatin with DNA is an increase in adduct repair and a decrease in the induction of apoptosis (Siddik 2003). Among the significant cellular pathways that have been demonstrated to play key roles in platin-induced apoptosis/cytotoxicity, the mitogen-activated protein kinase (MAPK) cascades, and the tumor suppressor p53 (Manic, Gatti et al. 2003; Sedletska, Giraud-Panis et al. 2005). Deciphering the mechanisms involved in chemoresistance is critical to improve our understanding of these complex pathways and to develop more effective targeted treatments.

Tumor cells often respond to chemotherapy by engaging protective mechanisms and survival signaling, which can antagonize the chemotherapy (Mayo and Baldwin 2000). Furthermore, apoptosis inhibition is necessary to provide cancer cells with the ability to survive in a stressful environment; it has been proposed that oncogenes provide cancer cells with intrinsic resistance. Chemoresistance in several types of cancer has been linked to the up-regulation of Bcl2 (Fesik 2005). Overexpression of Bcl2 is associated with cisplatin resistance, and this is likely facilitated by an increase in GSH levels (Hockenbery, Oltvai et al. 1993; Chiao, Carothers et al. 1995). However there are paradoxical findings, which indicate that Bcl2 overexpression is associated with either improved survival of ovarian cancer patients receiving cisplatin (Herod, Eliopoulos et al.

1996) or increased sensitivity of tumor cells to cisplatin (Beale, Rogers et al. 2000). So far, however, it has not been investigated whether overexpression of Bcl2 that is observed in smokers with NSCLC, is only an epiphenomenon or whether it is mechanistically involved in the development of drug resistance.

Cigarette smoking has been found to induce a number of genetic and molecular changes in the respiratory tract, including cellular atypia, loss of heterozygosity (Powell, Klares et al. 1999), and promoter hyper methylation (Guo, Akiyama et al. 2004). Microarray studies of bronchial epithelial cells have indicated that cigarette smoke induces, primarily, the expression of xenobiotic-metabolizing and redox-regulating genes but also points to the effects on tumor suppressor genes, oncogenes, and genes involved in the regulation of inflammation (Spira, Beane et al. 2004). Systematic analysis of mRNA and protein expression levels among thousands of genes has also contributed to defining the molecular network of lung carcinogenesis. Despite the fact that enormous progress in understanding the molecular mechanisms leading to lung cancer has been made, very little attention has been paid to how these molecular changes affect chemotherapy in smokers.

The transforming growth factor-beta (TGF-β) pathway has important roles in cellular proliferation, angiogenesis, differentiation, migration and apoptosis. The TGF-β pathway signal either through the SMAD-dependent pathway or SMAD independent pathway such as extracellular signal-regulated kinase, c-jun N-terminal kinase, PI3K/AKT and Rho-like guanosine triphosphatases. Despite the prominent role TGF-β pathway appears to play in regulating multiple cellular processes, there have not been any

studies elucidating the effect of this pathway on the resistance to chemotherapy in lung cancer.

Recent reports show that nicotine inhibits apoptosis in various cells lines, which may suggest that nicotine has the ability to reduce the efficacy of chemotherapeutic agents by stimulating survival pathways (Heeschen, Jang et al. 2001; Minna 2003; Dasgupta, Kinkade et al. 2006). In addition to nicotine, a burning cigarette generates as many as 6000 other compounds, amongst which 60 of them are known to be carcinogens. Although the toxicity of individual components can be assessed, it is likely that the biological response to complex mixtures such as cigarette smoke is not the sum of multiple independent toxicities. The best methodology to mimic effect of smoking in humans in vitro, would be to treat human lung cell lines with CSC and examine how that affects chemotherapy in lung cancer patients.

In our previous publication, we found that the Smad3 pathway is down regulated in long-term CSC treated cells and it contributes to lung tumorigenesis. In that paper we found that reduced expression of Smad3 due to long-term CSC treatment made the cells resistant to apoptosis, we conjectured that the Smad-dependent TGF-β pathway might also contribute to the resistance to chemotherapy of lung cancer patients. To answer that question we determined the IC50 of Carboplatin in the long-term CSC treated cells and elucidated how this pathway contributes to the resistance to carboplatin.

Results

Long-term CSC treatment imparts Carboplatin resistance

In our previous study, we have shown that long-term CSC treatment increases cell viability by decreasing apoptosis through up-regulation of anti-apoptotic Bcl2. To test whether long-term cigarette smoking contributes to the resistance to Carboplatin-based chemotherapy in lung cancer, we analyzed the cytotoxic effects of carboplatin treatment on a various time points of CSC treated A549 (lung adenocarcinoma) and HPL1A (immortalized bronchial epithelial) cell lines using the MTT cell viability assay. The long-term CSC treated cells (300-day CSC treated) were much more resistant to carboplatin treatment compared to the corresponding control (300-day DMSO treated) cells. In A549, the I.C.50 of Carboplatin for long-term (300-day) CSC treated cells was 167.5 µM whereas the corresponding control (300-day DMSO) cells had an I.C.50 of 72.3 µM (Fig. 1A). We saw the similar effect in HPL1A cells. The IC_{50} of 300-day CSC treated cells was 131.7 µM, whereas the IC_{50} of 300-day DMSO treated cells was 55.0 µM (Fig. 1B). Additionally, we observed that the IC_{50} of the cell lines increased with the increasing time of treatment of CSC. Bim, a pro-apoptotic factor is a downstream target of Smad3. Since in our previous study we elucidated that Smad3 expression goes down in long-term CSC treatment, we wanted to examine whether the expression levels of Bim are affected. To answer the question, we performed immunoblot analyses with the cell lysates prepared from long-term CSC treated cells and control cells. There was no difference in the expression of Bim in the long-term CSC treated A549 and HPL1A cells (Fig. 1C). Resistance to the cytotoxic effects of carboplatin in the long-term CSC treated cells lines were evidenced by a plateau pattern at lower doses of treatment. Taken

81

A

A549	Mean I.C. $_{50}$ (μM)	± S.D.
300 CSC	167.5 *	22.16
300 DMSO	72.25	22.36

B

HPL1A	Mean I.C. $_{50}$ (μM)	± S.D.
300 CSC	131.67 *	7.66
300 DMSO	55	8.66
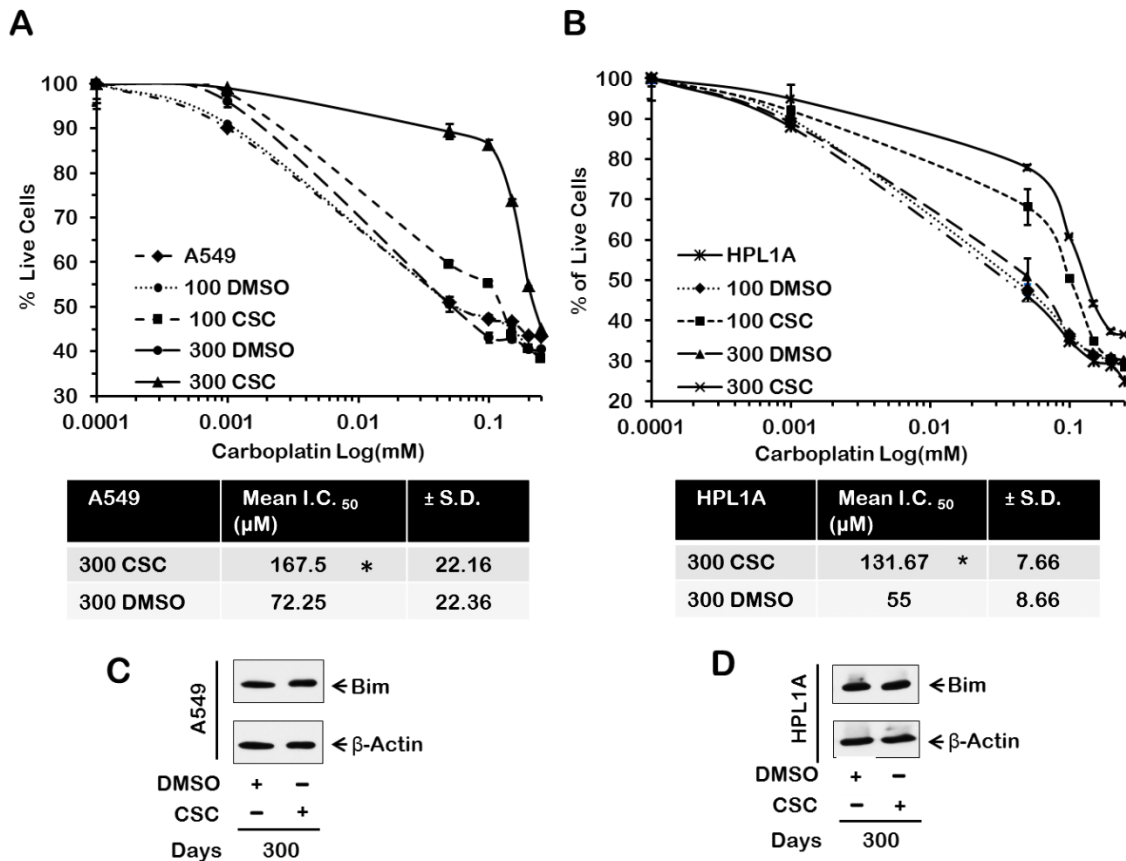
Figure 29. Long-term CSC treatment makes the cells resistant to Carboplatin due to up-regulation of Bcl2

A, B. 300-day CSC treated cells and 300-day DMSO treated (control) A549 (A) and HPL1A (B) cells were treated with Carboplatin for 72 hours, and cell viability was assessed as measured by MTT activity. Data are represented as a percentage of MTT activity where untreated cells were taken to be 100%. The I.C.50 and the corresponding S.D. were calculated from 6 individual experiments.

C, D. Bim expression 300-day CSC treated samples and controls in (C) A549 and (D) HPL1A cells by Western Blot analysis. β-Actin was used as a loading control.

together, these results may suggest that long-term CSC treatment makes the cells resistant to carboplatin-based chemotherapy.

CSC treatment induces resistance to Carboplatin by abrogating apoptosis

It is known that in many cases the cells become resistant to chemotherapeutic drugs due to less induction of apoptosis by drugs. To test whether apoptosis is playing a role in CSC induced resistance, we conducted apoptosis studies by FACS analyses. 300-day DMSO and CSC treated cells were treated with fixed concentration of Carboplatin for 48 hours and then analyzed for apoptosis induced by Annexin V-PI staining. We observed that in the control cells Carboplatin induced a higher percentage of cell death (28 for HPL1A and 20 for A549) compared to the corresponding CSC treated cells (4 for HPL1A and 3 for A549) (Fig. 30 B and D). Thus, the long-term CSC treatment abrogated carboplatin-mediated apoptosis in both cell lines. To confirm the induction of apoptosis by another method, we performed immunoblot analysis of cleaved PARP, another hallmark of apoptosis, on the samples with increasing concentration of Carboplatin for 48 hours. We observed a dose dependent increase in PAPR cleavage in the DMSO treated A549 and HPL1A cells. CSC treated cells did not show any PARP cleavage (Fig. 30 C and E). Carboplatin often cause cell cycle arrest in the G2/M phase. An arrest of the cells in the G2/M phase is seen as inhibitory to the cytotoxic processes of the drug. To test whether long-term CSC treatment causes the cells to be arrested more in the G2/M phase, in response to Carboplatin, we performed cell cycle analysis using FACS. Cells were treated with Carboplatin for 48 hours and the percentages of cells having 2N DNA content were quantitated (as a measure of G2/M phase). We observed that in the long-term CSC treated cells the carboplatin-induced arrest in the G2/M phase was higher than in the

corresponding control cells (Fig. 31). Taken together, these results suggest that long-term

CSC treatment abrogates carboplatin-induced apoptosis by arresting the cells in the G2/M

phase of the cell cycle.
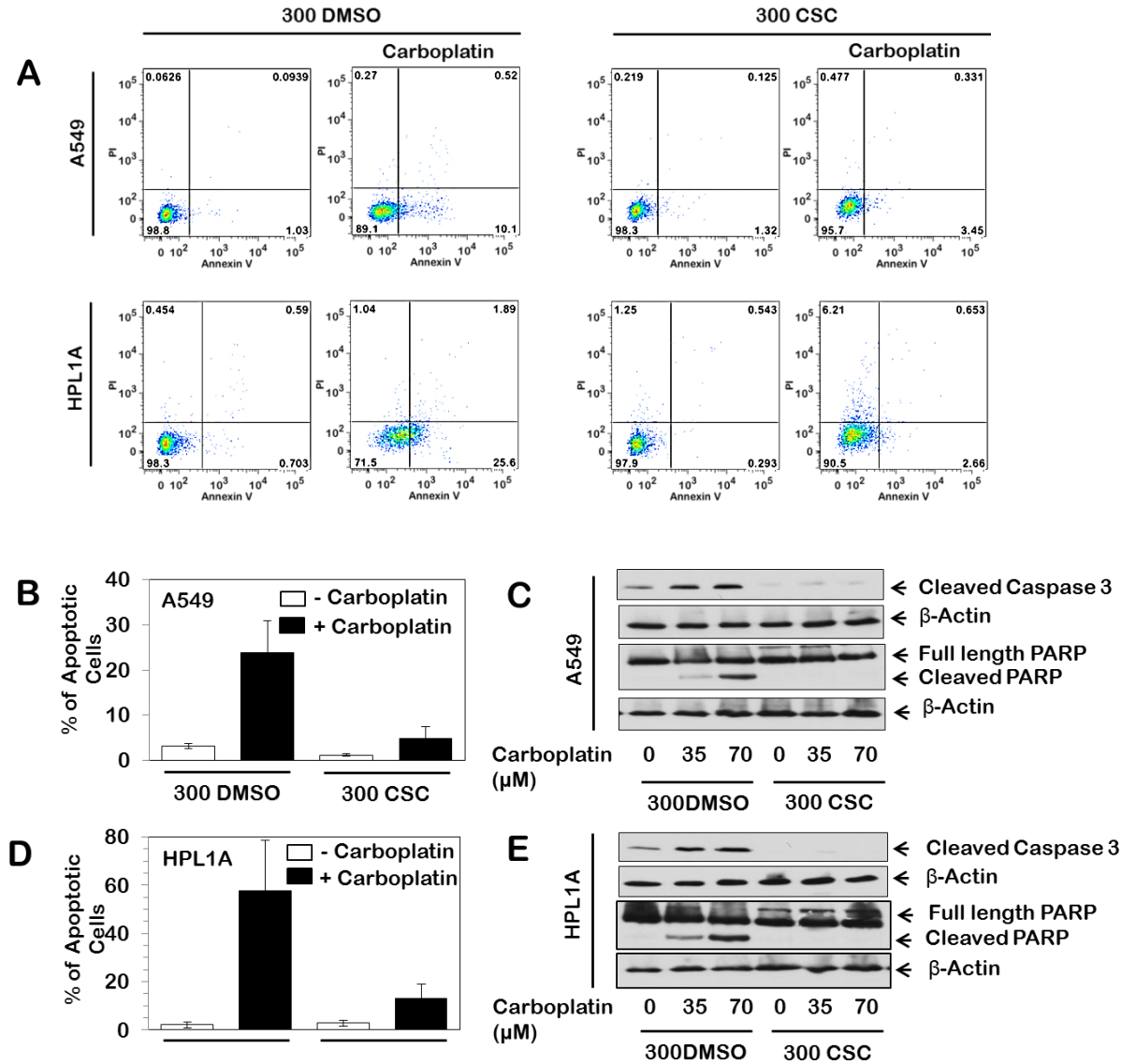
Figure 30. Long-term CSC treatment makes cells resistant to Carboplatin through the abrogation of apoptosis.

A. Representative of figures of Apoptosis detected by Annexin-V-PI staining on 300 day CSC treated and control for both A549 and HPL1A cells treated with carboplatin for 48 hours.

B, D. Quantitation of apoptosis detected by Annexin V-PI staining on the 300-day CSC

treated and control for both A549 and HPL1A cells treated with Carboplatin 70 µM for

72 hours analyzed by FACS analysis. Quantitation and statistical analysis were calculated

from 4 individual experiments.

C,E. Cleaved-PARP and Cleaved Caspase-3 expression in 300-day CSC treated and

control A549; HPL1A cells after treated with increasing concentration of Carboplatin at

35 µM and 70 µM for 48 hours. β-Actin was used as loading control.
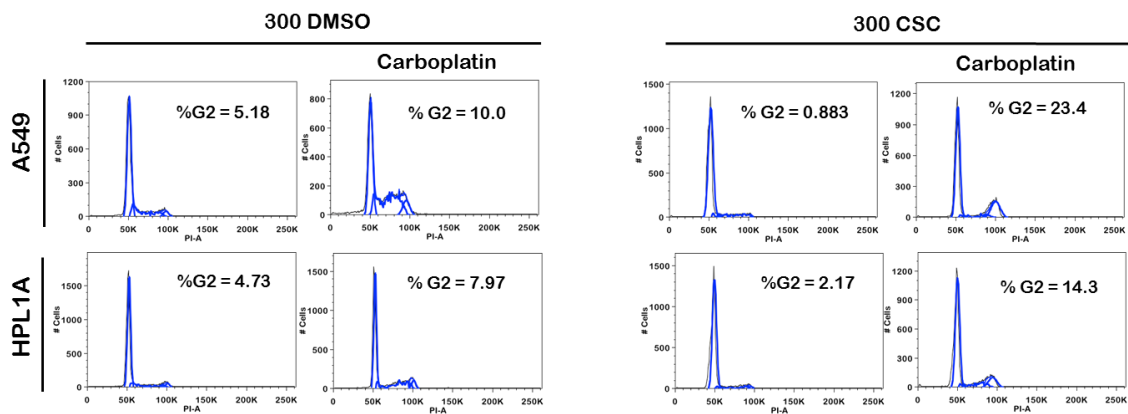
**Figure 31. Long-term CSC treatment abrogates Carboplatin mediated apoptosis by
arresting the cells in G2 phase of cell cycle.**

 Cell cycle analysis of 300-day CSC treated cells and control cells (300-day DMSO) after

treatment with Carboplatin for 48 hours. Figure is a representative of four different

experiments.

Bcl2 is important for the inhibition of Carboplatin-induced apoptosis

CSC contains thousands of compounds, comprised of five known human carcinogens and many toxic agents. These may affect different pathways ultimately leading to increased carboplatin resistance. To test whether Bcl2 is involved in CSC induced chemoresistance, we performed rescue experiments by knocking down Bcl2 in long-term CSC treated cells by siRNA. We transfected Bcl2 siRNA and a control scrambled siRNA in the long-term CSC treated cells (both A549 and HPL1A). The next day, cells were treated with Carboplatin for 48 hours and checked for apoptosis by FACS analysis. Immunoblot analyses were performed at the same points to verify the knockdown of Bcl2 in the long-term CSC treated cells (Fig. 32A and B, upper panels). In support of our hypothesis, we observed that on knocking down Bcl2 in the long-term CSC treated cells there is a significant increase in the induction of apoptosis in both the cell lines. These results suggest that Bcl2 is important for the long-term CSC treated cells to become resistant to carboplatin-mediated apoptosis. To confirm that Bcl2 is sufficient to make the cells resistant to carboplatin, we overexpressed exogenous Bcl2 in the parental A549 and HPL1A cells where the expression of Bcl2 is low (Fig. 33 A and B, upper panels). To test whether overexpression of Bcl2 decreases the sensitivity to Carboplatin mediated apoptosis, we treated the Bcl2 overexpressing clones along with parental and vector controls with Carboplatin for 48 hours. We observed that the Bcl2 overexpressing clones were resistant to Carboplatin mediated apoptosis compared to the parental and vector controls (Fig. 33 A and B, lower panels). We then determined the I.C.50 of Carboplatin in both the clones stably overexpressing Bcl2 along with the vector controls. Stable overexpression of Bcl2 in the parental A549 and HPL1A cells increased the I.C.50 of
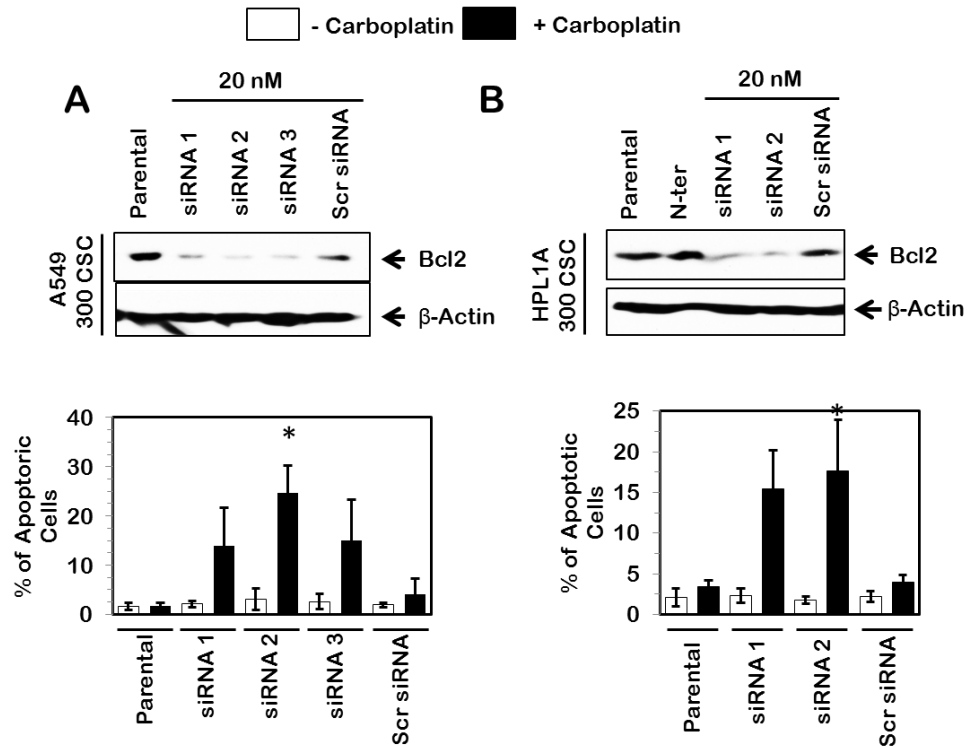
Figure 32. Bcl2 is involved in making cells resistant to Carboplatin.

A.   Bcl2 expression in the 300-day CSC treated A549 and HPL1A cells transfected

with siRNA directed against Bcl2 and scrambled control. β-Actin was used as a loading

control.

B.   Percentage of apoptotic cells detected by Annexin V-PI staining in the 300-day

CSC treated cells after no treatment (white) or after treatment with 70 µM Carboplatin

(black) for 48 hours after transfection with siRNA against Bcl2 and scrambled control.

Statistical analyses were calculated based on 4 individual experiments.
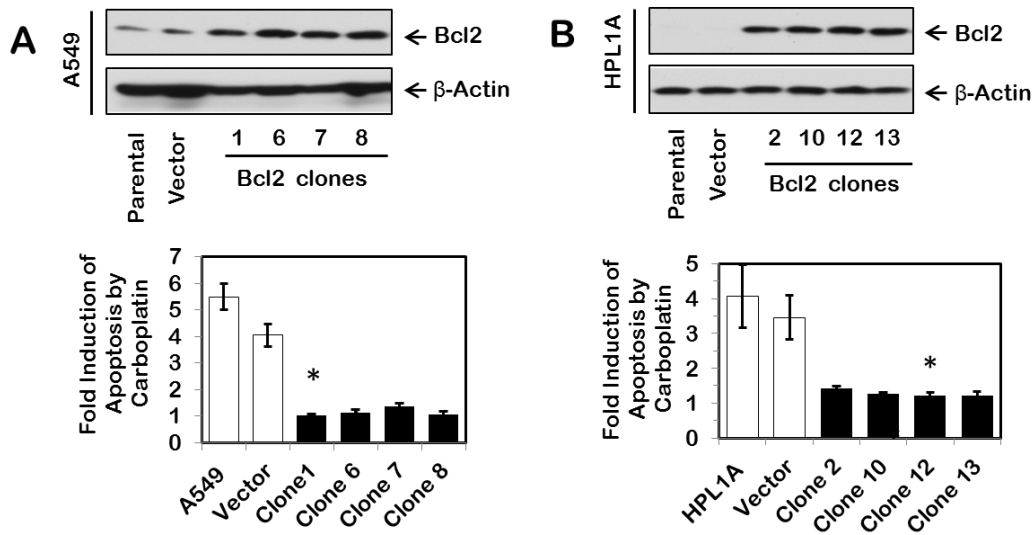
Figure 33. Overexpression of Bcl2 makes the cells resistant to Carboplatin induced apoptosis.

A, B. Bcl2 expression in the Bcl2 overexpressing clones in the (A)A549 and (B) HPL1A cells. β-Actin was used as a loading control.Fold induction of apoptosis by Annexin V-PI staining in parental, Bcl2 overexpressing clones, and vector control after treatment of 70 μM Carboplatin compared to no treatment of Carboplatin in the corresponding clones for 48 hours. Statistical analyses were calculated based on 4 individual experiments.
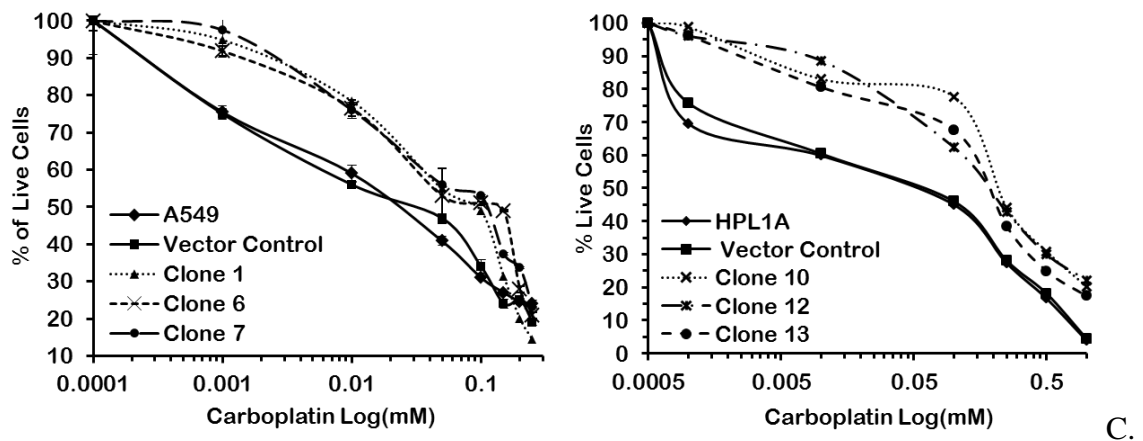
Figure 34. Over-expression of Bcl2 increases the I.C.50 of Carboplatin

Parental A549 (left panel), HPL1A (right panel), Bcl2 overexpressing clones and vector controls were treated with varying concentration of Carboplatin for 72 hours and assayed for cell viability as measured by MTT activity. The I.C.50 was calculated as described in Materials and Methods. Standard deviations were calculated based on triplicate data points for each concentration. Statistical analyses were calculated based on 3 individual experiments.

Carboplatin in both cell lines (Fig. 34). The average I.C.50 of Carboplatin for Bcl2 overexpression clones were 100 µM for A549 and 125 µM for HPL1A; whereas the average for the parental and vectors were 25 µM for A549 and 50 µM for HPL1A. Taken together, these experiments suggest that Bcl2 is necessary and sufficient for long-term CSC treated cells to make them resistant to Carboplatin induced apoptosis.

ABT-737 sensitizes the long-term CSC treated cells to Carboplatin

Numerous examples exist in the literature of an enhanced apoptotic response when the BH3 mimetics are combined with traditional therapies to treat various cancers such as melanoma, pancreatic, glioma, breast, multiple myeloma, and B-cell malignant models (Witham, Valenti et al. 2007; Shoemaker, Mitten et al. 2008; Tse, Shoemaker et al. 2008). We wanted to test whether BH3 mimetic ABT-737 would sensitize the cells to Carboplatin in our in vitro model system. First, we tested the effect of the ABT-737 on apoptosis in our model system. We treated the 300-day CSC treated cells and the control cells with increasing concentration of ABT-737 for 48 hours and assayed for apoptosis. We observed that ABT-737 induced more apoptosis in the 300-day CSC treated cells compared to the control cells in both A549 and HPL1A cells (Fig. 35 A and B, upper panels). The sensitivity of ABT-737 depends on the expression of another anti-apoptotic Bcl2 family protein, Mcl1 (van Delft, Wei et al. 2006). To test whether increased levels of Mcl1 is contributing to the increased resistance to ABT-737, we performed immunoblot analysis on the samples. We observed that the expression of Mcl1 does not change due to CSC treatment (Fig. 35A and B, lower panels). We can conclude that the
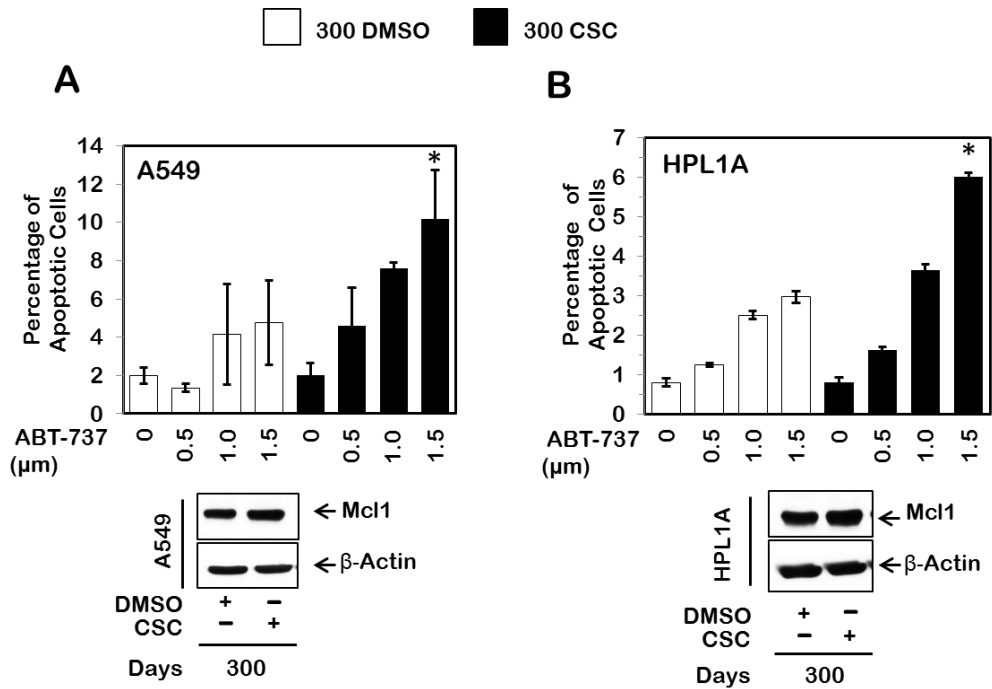
Figure 35. Long-term CSC treated cells are more sensitive to ABT-737.

A,B.   300-day CSC treated (300-day DMSO as a control) A549 (A) (upper panel) and HPL1A (B) (upper panel)cells were treated with increasing concentration of ABT-737 for 48 hours and percentage of cells undergoing apoptosis were detected by Annexin V-PI staining. Statistical analyses were calculated based on 4 individual experiments. Cell lysates from 300-dat CSC treatment and controls were analyzed by immunoblot analysis with pro-apoptotic anti-Mcl1 antibody. An equal amount of protein loading was verified by immunoblot analysis with anti-β-actin antibody (lower panels).
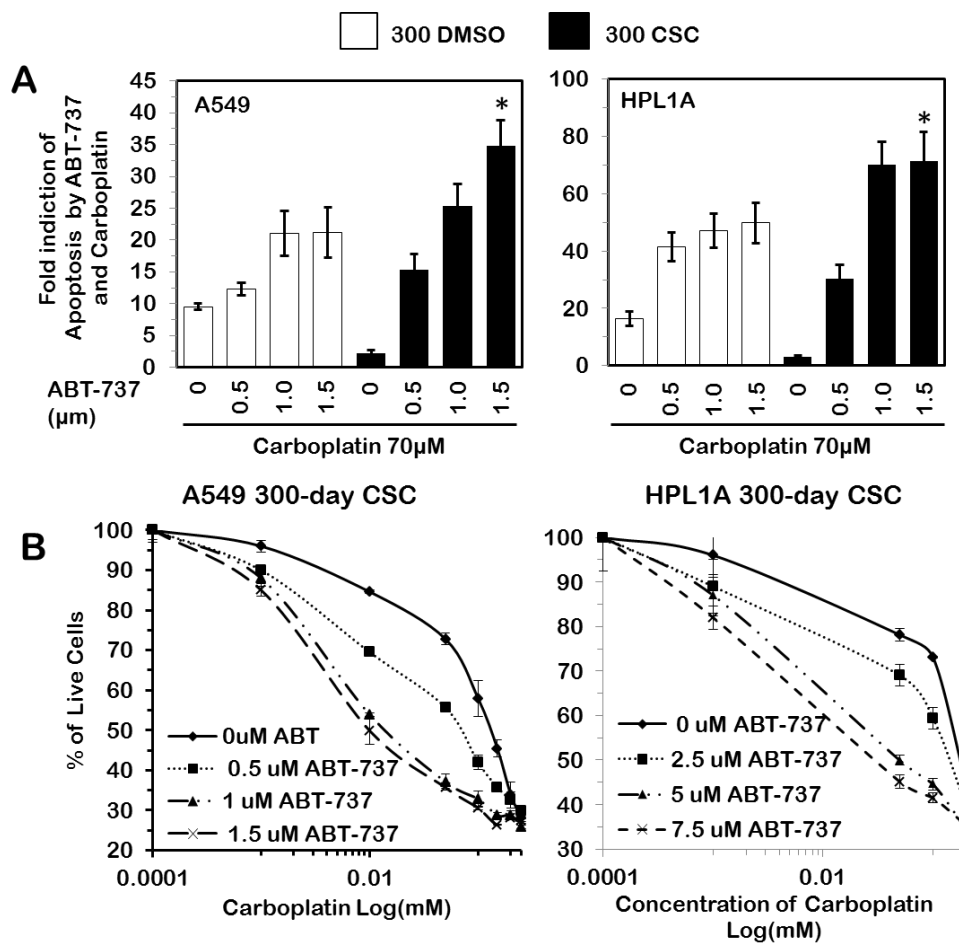
Figure 36. ABT-737 resensitizes the long-term CSC treated cells to carboplatin.

A.   Fold induction of apoptosis in 300-day CSC treated A549 and HPL1A cells along

with control cells were treated with or without 70 μM carboplatin and increasing

concentration of ABT-737 (0-1.5 μM) assayed by Annexin V-PI staining. Fold induction

was calculated from the ratio of percentage of cells undergoing apoptosis undergoing

treatment with respect to the apoptosis in the untreated samples. Error bars are

representative of four independent experiments.

B.   300-day CSC treated cells treated with carboplatin (0-250 μM) in the presence of

increasing concentration of ABT-737 0;0.5;1.0;1.5 μM for A549 and 0;2.5;5;7.5 μM for

HPL1A cells for 72 hours were assessed for cell cytotoxicity as measured by MTT activity. The data are presented as a percentage of MTT activity where untreated cells were taken to be 100%. Error bars are representative of four independently treated samples.

increased sensitivity of ABT-737 is not due to increased expression of Mcl1. Next, we wanted to test whether ABT-737 can sensitize the 300-day CSC treated cells selectively to Carboplatin mediated apoptosis. To test this hypothesis, we treated 300-day CSC treated cells along with control cells with same concentration of Carboplatin, and increasing concentration of ABT-737 for 48 hours and assayed for apoptosis by FACS analyses. We observed that the fold induction of apoptosis on top of Carboplatin by ABT-737 was higher in the 300-day CSC treated cells compared to the control cells (Fig. 36 A). Next, we wanted to test whether the ABT-737 could sensitize the 300-day CSC treated cells to carboplatin. We determined the I.C.50 of Carboplatin with increasing concentrations of ABT-737. We observed that ABT-737 decreased the I.C.50 of Carboplatin in both the cell lines in a dose-dependent manner (Fig. 36 B). Taken together, these results prove the niche of ABT-737 to be used in combination with Carboplatin to improve the therapeutic efficacy.

Smad3 induces sensitivity of Carboplatin in long-term CSC treated cells by down-

regulating Bcl2

In our previous studies, we have shown that the expression of Smad3 decreases in the long-term CSC treated cells. We also showed that it is associated with the increased expression of Bcl2. To test whether Smad3 can alter the sensitivity of long-term CSC, we stably overexpressed Smad3 in the 300-day CSC treated A549 and HPL1A cells. We then verified the overexpression of Smad3 in the 300-day CSC treated cell by Western blot analyses (Fig 37A, upper panel). To test whether Bcl2 is regulated by Smad3, we performed Western blot on the Smad3 overexpression clones to check the expression of Bcl2. We observed that Bcl2 expression is reduced in the Smad3 overexpression clones (Fig 37A, middle panel). Next, we wanted to test whether re-expression of Smad3 can re-sensitize the cells to carboplatin-mediated apoptosis. We treated the Smad3 overexpressing clones, vector control and the 300-day CSC treated cells (both A549 and HPL1A) with Carboplatin for 48 hours and assayed for apoptosis. We observed that Smad3 overexpressing clones showed a much higher fold induction in apoptosis than the 300-day CSC treated and the vector controls (Fig. 37B). We extended the results further and tested whether the Smad3 overexpression can actually sensitize (decrease the I.C.50)
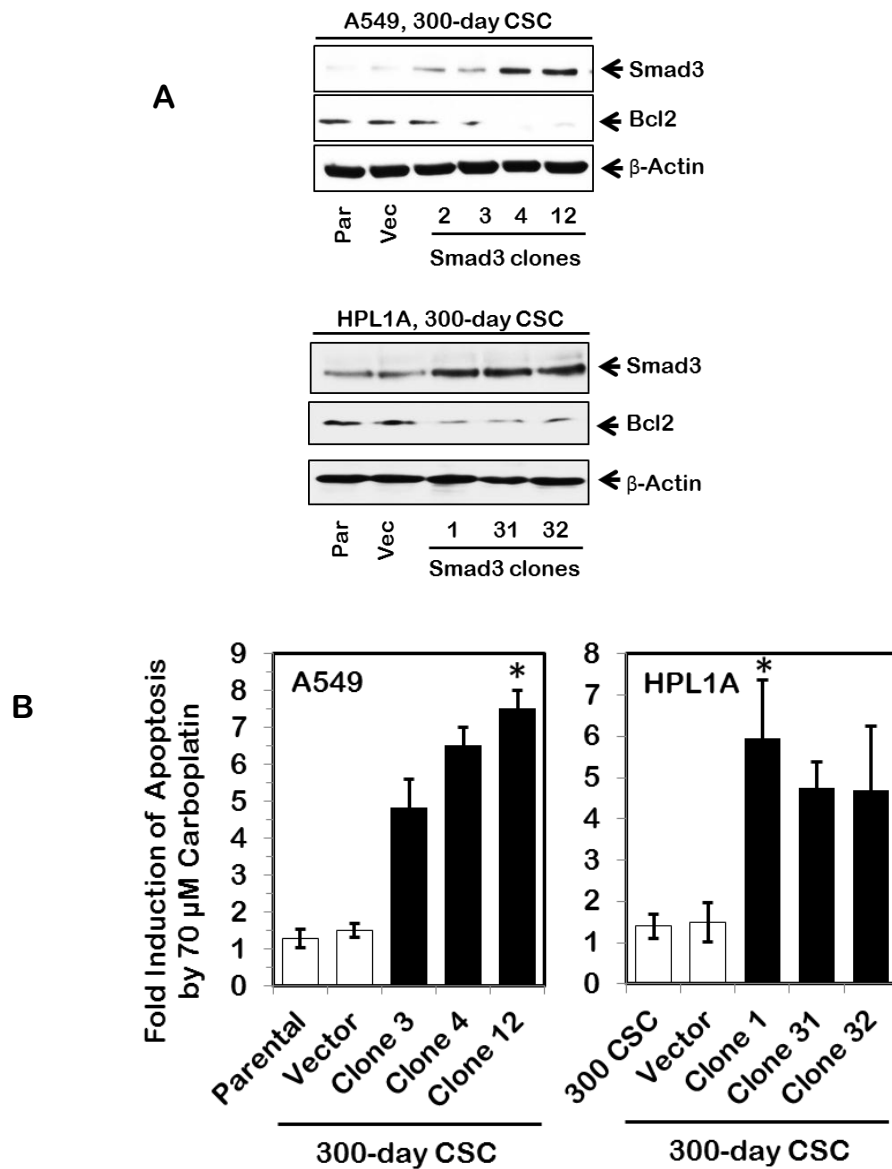
Figure37. Smad3 expression mediates, in part, the cytotoxic effects of carboplatin.

A.   Protein expression of Smad3, Bcl2 in Smad3 overexpressing clones, vector control in the 300-day CSC treated A549 and HPL1A cells. An equal amount of protein loading was verified by immunoblot analysis with anti-β-actin antibody.

B.   Fold induction of apoptosis in the 300-day CSC treated cells, vector controls and

Smad3 overexpressing clones after treatment of 70 μM carboplatin for 48 hours. Fold

induction was calculated from the ratio between percentage of cells undergoing apoptosis

with treatment and without treatment of carboplatin in the corresponding cells. Error bars

were calculated from four individual experiments.
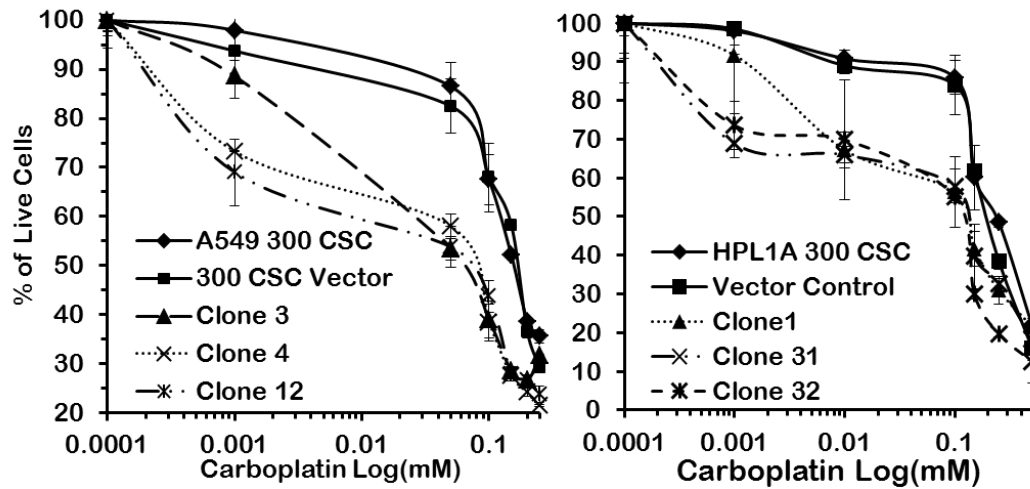
Figure 38. Re-expression of stable Smad3 resensitizes the long-term CSC treated

cells to Carboplatin

300-day CSC treated A549 and HPL1A, Smad3 overexpressing clones and vector

controls were treated with varying concentration of Carboplatin for 72 hours and assayed

for cell viability as measured by MTT activity. The data are presented as a percentage of

MTT activity where untreated cells were taken to be 100%. Error bars are representative

of four independently treated samples.

the 300-day CSC treated cells to Carboplatin. We determined the I.C.50 of Carboplatin in the Smad3 overexpressing clones. We observed that the I.C.50 of Carboplatin was less in the Smad3 overexpressing clones compared to the parental (300-day CSC treated) and the vector controls in both A549 and HPL1A cell lines (Fig. 38). The average I.C.50 of Carboplatin for Smad3 overexpression clones were 85 µM for A549 and 100 µM for HPL1A; whereas the average for the parental and vector were 130 µM for A549 and 150 µM for HPL1A cells. Taken together, these results suggest that Smad3 regulates the expression of Bcl2 and, in turn, controls the sensitivity of the 300-day CSC treated cells to Carboplatin.

Loss of Smad3 expression in CSC treated cells induces resistance to Carboplatin by the up-regulation of Bcl2

Smad3 has many downstream targets, and overexpression of Smad3 can regulate other pathways that may contribute to the increased sensitivity other than the down-regulation of Bcl2. We hypothesize that the sensitivity to Carboplatin mediated apoptosis in the Smad3 overexpressing clones is exclusively due to down-regulation of Bcl2 and not some off target effects. To test this hypothesis, we used Smad3 siRNA to suppress Smad3 expression in the Bcl2 overexpressing clones in both A549 and HPL1A cells. Immunoblot analysis performed in parallel experiments demonstrated the successful knockdown of Smad3 in the Bcl2 overexpression clones (Fig. 39A and B, upper panels).
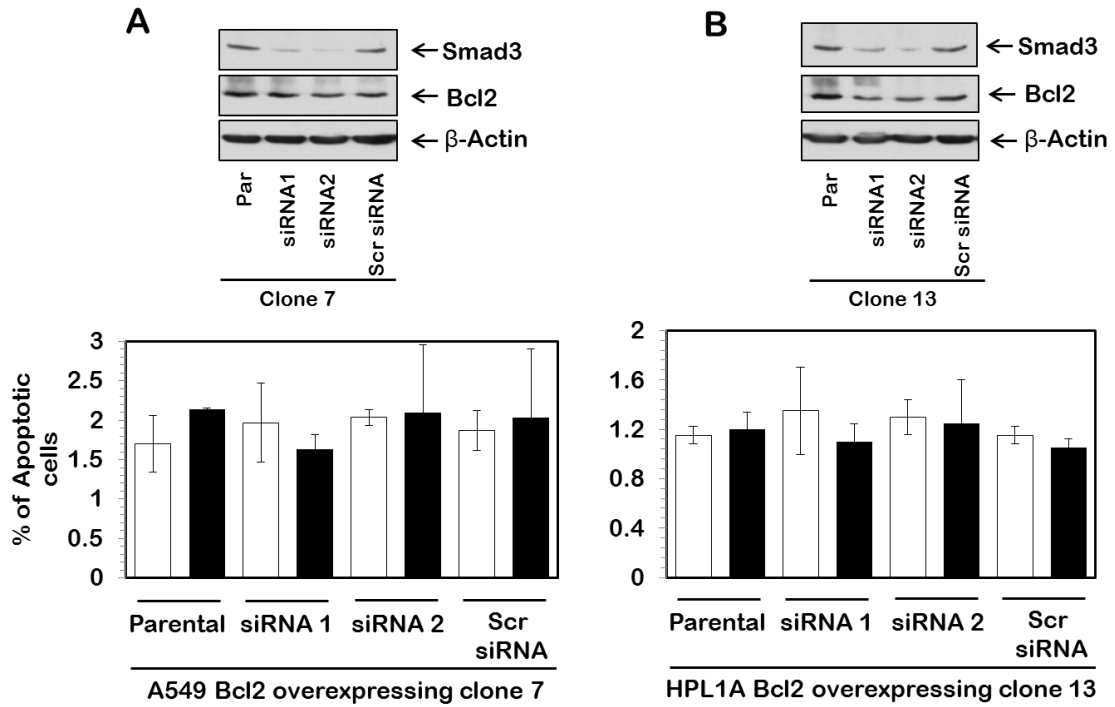
Figure 39. Smad3 makes the cells resistant to Carboplatin mediated apoptosis specifically through the Bcl2 pathway.

A. Expression of Smad3, Bcl2 in the Bcl2 overexpressing clones of A549 and HPL1A cells after transfection of Smad3 siRNA and scrambled control by Western Blot. β-Actin was used as a loading control.

B. Percentage of cells undergoing apoptosis in the Bcl2 overexpressing clones in A549 and HPL1A cells, after transfected with Smad3 siRNA and scrambled control and treated with carboplatin 50 μM for 48 hours. Error bars are calculated from four individual experiments.

The loss of Smad3 in the Bcl2 overexpressing clones did not induce apoptosis by

Carboplatin treatment for 48 hours in both the cell lines (Fig 39A and B, below panels).

Taken together, these results demonstrate that loss of Smad3 expression induces

resistance to carboplatin in 300-day CSC treated cells specifically through Bcl2.

Correlation between Smad3 and Bcl2 expression in lung tumors

In our in vitro model system, we observed that Smad3 regulates the expression of Bcl2.

Since it is an in vitro model system, it might be an artifact of long-term cells in culture.

To verify whether the inverse correlation between the expression of Smad3 and Bcl2 is

valid in lung tumors or not, we looked into the public database (Shedden, Taylor et al.

2008) to investigate the correlation between Smad3 mRNA expression and Bcl2 mRNA

expression. We found that lung cancer patients with higher levels of Smad3 have lower

levels of Bcl2 and patients having lower levels of Smad3 have relatively higher levels of

Bcl2 mRNA expression (Fig. 40). The results were statistically significant. This data

suggest that an inverse correlation exists between the mRNA expressions of Smad3 and

Bcl2 in lung tumors.
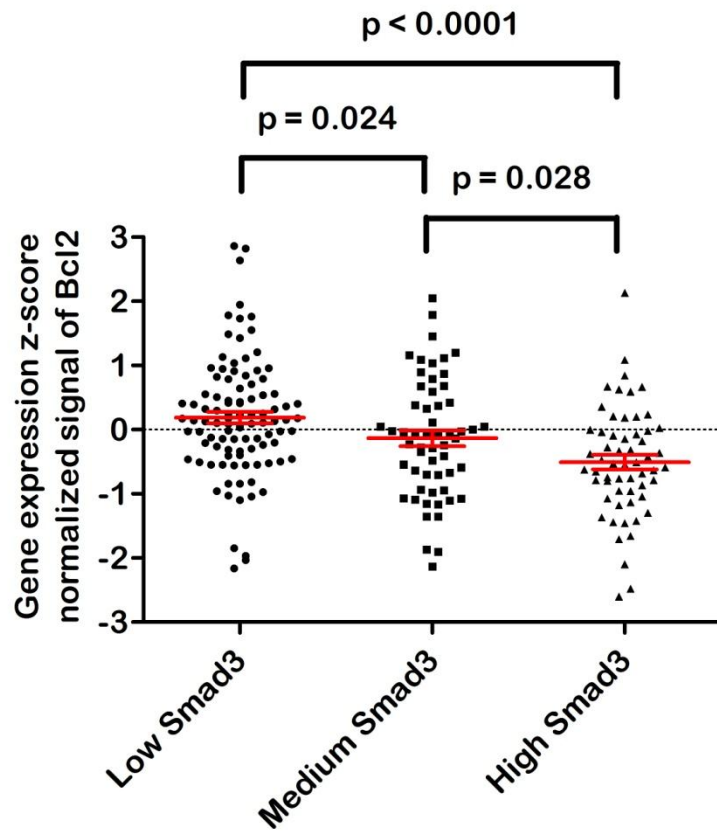
Figure 40. Smad3 and Bcl2 expressions are inversely correlated in non-small cell lung cancer.

Box plot of Bcl2 expression as a function of Smad3 expression status in patient tumor samples from public database. p values calculated by student t-test.

Discussion

In this paper, we have used an in vitro system to mimic long-term smoking in human. We treated a tumor cell line (A549) and an immortalized bronchial epithelial cell line (HPL1A) with Cigarette smoke condensate (CSC) for various time points of treatment. Since the CSC is dissolved in DMSO, we also treated the cells with DMSO as a control, to take into account, if any, of the effects of long-term cell culture. We demonstrated that long-term CSC treatment makes the cells resistant to carboplatin, and Smad3 is partly responsible for making the cells resistant to chemotherapy. To our knowledge, this is the first time Smad3 has been shown to be responsible for creating resistance to chemotherapy in lung cancer. We also show, for the first time, that in NSCLC, there is an inverse correlation between the expression of Smad3 and Bcl2. Smad3 is a regulator of Bcl2 in lung cancer.

Although progress has been made in the field of cancer treatment, no method is 100% effective for success. One major obstacle in cancer treatment is the resistance of cancer cells to anticancer drug therapies. In fact, drug resistance is currently the most common factor in tumor recurrence. Resistance to therapy can be classified by two categories: intrinsic and acquired. Intrinsic resistance implies that prior to receiving the intended therapy; there already exists (de novo) factors that would make the intended therapy ineffective. During the course of treatment, tumors, that are not initially resistant to a particular drug, often quickly develop acquired resistance. This is because of the prevailing selection and overgrowth of drug-resistant variants in the tumor cells, resulting in the futility of that treatment (Blagosklonny 2004). Correctly selected targeted treatments are guaranteed to improve tumor response rates. The results from our current

study serve as examples of the utility of the in vitro model for defining the subset of patients with favorable outcomes in cell line systems. After appropriate preclinical validation, these suggestions are expected to lead to improved outcomes in clinics. According to our data, lung cancer patients who are/were long-term smokers are intrinsically resistant to carboplatin-based chemotherapy.

Stabilization and activation of wild type p53 are critical for cisplatin-mediated apoptosis. Therefore, the tumor cells devoid of the wild type p53 function fail to activate the cell death program. Thus, the cells become tolerant to DNA damage, which is a feature characteristic of resistance caused by disruption in signal transduction pathways (Kastan, Onyekwere et al. 1991; Hartwell and Kastan 1994; Pietenpol, Tokino et al. 1994; Siddik, Mims et al. 1998). However, the NCI panel of cell lines demonstrates a wide range of overlapping responses to cisplatin for the group of wild type and mutant p53 tumor models, with some mutant p53 models expressing exquisite cisplatin sensitivity (O'Connor, Jackman et al. 1997). These observations have also been documented in clinical cases, where tumors demonstrate either sensitivity or resistance to cisplatin irrespective of the p53 gene status (Righetti, Tosi et al. 1996). Similar to these observations, we also observed that the resistance to cisplatin is irrespective of the status of p53 (Fig. 29). In HPL1A cells, p53 is inactivated due to immortalization by SV-40 large T-antigen, whereas the other cell line A549 has wild type active p53.
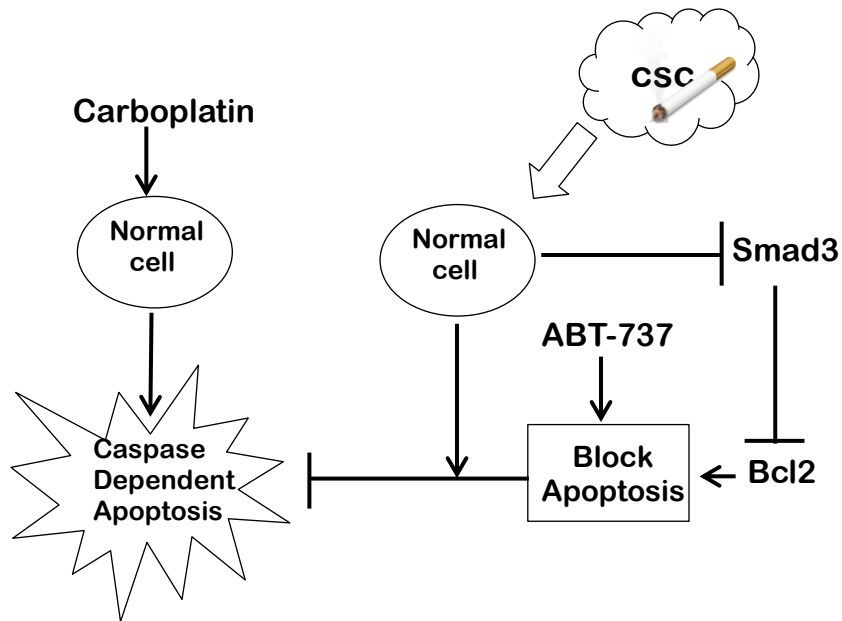
Figure 41. Model for CSC treatment induced resistance to carboplatin .

In normal lung cells carboplatin induces caspase-dependent apoptosis. Long-term

CSC treatment results in reduced expression of Smad3, which results in the up-

regulation of anti-apoptotic Bcl2 which creates resistance to carboplatin by blocking

caspase-dependent apoptosis. This long-term CSC treated cell can be resensitized

with BH3 mimitic, ABT-737.

In spite of this difference in p53 status, long-term CSC treatment makes the cells resistant to carboplatin treatment. Another genetic factor which might contribute to the sensitivity to carboplatin is K-ras which can activate various pathways namely PI3K/Akt, MAPK. A549 has a mutation in the K-ras, which makes it constitutively active. However, we saw carboplatin resistance in both the A549 and HPL1A cells. These studies demonstrate that the sensitivity to carboplatin is irrespective of the K-ras mutation status.

The relationship between cell cycle arrest and cytotoxicity is complex and not fully deciphered. According to the current dogma, cell cycle arrest is seen as inhibitory to the cytotoxic process. This conclusion is derived primarily from the demonstration that pharmacological abrogation of the G2/M checkpoint increases cellular sensitivity to cisplatin (Demarcq, Bunch et al. 1994; O'Connor and Fan 1996). This is consistent with the concept that in response to DNA damage, the cell undergoes cell cycle arrest to enable the nucleotide excision repair (NER) complex to remove the adducts and thus promote cell survival. Cells undergo apoptosis only when repair is incomplete by excessive DNA damage. Similarly, we found that the long-term CSC treatment makes the cells arrest in the G2/M phase of cell cycle and as a result exhibited decreased apoptosis (Fig. 30 and 31). This could be due to the up-regulation of Bcl2. Recently, it has been shown that overexpression of Bcl2 is responsible the arrest of the cells in the G1/G2 phase through the induction of cyclin-dependent kinase inhibitors namely, p27, p21, p16. We also demonstrated that the overexpression of Bcl2 that is observed in smokers with NSCLC, is not only an epiphenomenon but also is mechanistically involved in the development of carboplatin resistance (Fig. 32,33 and 34).

It has been shown that ABT-737, a BH3 mimetic, binds to anti-apoptotic Bcl2 proteins and disrupts the sequestering and neutralizing of pro-apoptotic proteins namely, Bim and Bid. The drug has shown efficacy on administration as both a mono therapy and in combination with cytotoxic therapies. Even in our long-term CSC treated cell lines ABT-737 induced apoptosis, the putative reason for little induction of apoptosis may be due to the relatively high expression of Mcl1 in the cell lines (Fig.35A and B, lower panels). The sensitivity of ABT-737 to long-term CSC treated cells is not due to the decreased expression of anti-apoptotic Mcl1 as shown previously in some model systems. The differential sensitivity is possibly due to the high expression of Bcl2 from long-term CSC treatment as shown in previous studies (Tahir, Yang et al. 2007; Placzek, Wei et al. 2010). The other niche for these drugs is in combination therapy where the Bcl2 antagonist serves to inhibit Bcl2-mediated resistance, enabling killing by conventional chemotherapy. In congruence to the previous studies, we illustrate that in our in vitro model (ABT-737) can improve the efficacy of Carboplatin. There is a strong indication that ABT-737 synergizes with Carboplatin, which is also in congruence to previous studies (Fig.36).

In our previous study we showed that long-term CSC treatment caused a reduced expression of Smad3 and that caused the decrease in apoptosis. Our current results show that Smad3 regulates the expression of Bcl2 in our in vitro model system (Fig. 37A). This is in accordance to a previous publication showing Smad3 regulates the expression of Bcl2 in hepatocellular carcinoma (Yang, Zhang et al. 2006). Further, the regulation of Bcl2 expression levels by Smad3 has a causal effect of decreasing the I.C.50 of carboplatin by increasing the carboplatin-mediated apoptosis (Fig. 37B and 38). This is

also in agreement with in vivo studies that have shown that blocking TGF-β signaling can result in resistance to DNA-damaging agents including cisplatin (Stoika, Yakymovych et al. 2003; Kano, Bae et al. 2007). Bim, a pro-apoptotic factor, is another target of Smad3 (Wildey, Patil et al. 2003). In our in vitro model we do not see any change in Bim levels (Fig. 29 C and D) even when Smad3 expression is reduced due to CSC treatment. It is quite possible that the regulation of Bim expression by Smad3 is context dependent. Along with the results (Fig. 39) and the observation that Bim levels are not changed by Smad3 in our model system, this demonstrates that Smad3 induces carboplatin resistance by regulating the expression of Bcl2. The in vitro results of the inverse relationship between the expression of Smad3 and Bcl2 also holds true in lung cancer patients at the mRNA level (Fig. 40). To our knowledge, this is the first time the inverse correlation between the two genes is shown in lung cancer (Fig. 40). It is worth exploring whether reduced expression of Smad3 and high expression of Bcl2 can serve as a prognostic or predictive biomarker for carboplatin based chemotherapy. Based on the observations, we propose a model that long-term CSC treatment induces an intrinsic resistance to carboplatin by upregulating the anti-apoptotic Bcl2 (Fig. 41). The upregulation of Bcl2 is induced by the reduced expression of Smad3. This intrinsic resistance to carboplatin induced by long-term smoking can be overcome by treating with BH3 mimetics, ABT-737. Additionally, from our data it would suggest that HDAC inhibitors namely, Sodium Buryrate may also resensitize the long-term CSC cells to carboplatin by restoring the expression of Smad3.

Contemporary first-line chemotherapy in advanced stage lung cancer traditionally involves platinum-based drugs. In lung cancer, resistance to cancer drugs presents a

major problem, limiting the effectiveness of chemotherapy. In the current era of personalized medicine, treatment selection for each cancer patient is becoming individualized or customized. There is a pressing need to identify predictive biomarkers for stratifying and subgrouping lung cancer patients for platinum-based therapy. Our results serve as examples of the utility of in vitro models for defining the subset of patients with favorable outcomes in cell line systems. After appropriate preclinical validation, these suggestions are expected to lead to a definitive subset of patients who would benefit from platinum-based chemotherapy or how the response of the resistant patients could be improved.

CHAPTER V

CONCLUSION AND FUTURE DIRECTION

Epidemiological studies have clearly established that tobacco products cause cancer of various types. Improving our understanding of the relevant carcinogenic mechanism will lead to new approaches for cancer prevention, early detection and perhaps improve therapy. Earlier diagnosis of patients with lung cancer would increase the number of potentially resectable patients, but better detection approaches are needed. Over the past two decades, several valuable tobacco-carcinogen biomarkers have been discovered, which have the potential to increase our mechanistic insights into lung carcinogenesis. Understanding the mechanisms by which smoking contributes to cancer has been and will remain, a major research focus. Several approaches have been taken, but none of them are full proof. Each system has its own tradeoffs.

One of the in vivo approaches is to expose animals to cigarette smoke using a smoking chamber for extended period of time. One inherent problem to this approach is that, animals do not smoke the way as humans do. Exposing the whole body animals to the smoking machines is more like passive smoke exposure than passive exposure. This is more of a "whole body exposure". Another problem is that other animal species do not breathe in the same manner as do people. Rodents for example are obligate nose breathers. So the pattern of particle filtration in the nares and upper respiratory tract would be different than the mouth breathing in case of humans. Thus, whereas use of

smoke is much more plausible than smoke extract as an exposure for animals in in vivo studies, but the model system imperfectly models human exposure.

Another approach has been to capture the smoke emitted from a cigarette by bubbling it through an aqueous solution. Such preparation is called the cigarette smoke condensate. The exposure of cells to cigarette smoke condensate has its own trade offs. First the smoke condensate is different from routine smoke, since the volatile and rapidly reactive components are lost. A variety of chemical changes can take place with storage. Secondly, in vivo cells are not exposed to "smoke" but rather to components of smoke that have been extracted to biological fluids. Although the toxicity of individual components can be assessed, it is likely that the biological response to complex mixtures such as cigarette smoke is not the sum of multiple independent toxicities.

Despite these noted methodologic limitations, studies using these techniques have provided great insight and are likely to continue to do so. Over the past decade there are plenty of publications which suggest cigarette smoke condensate is capable of activating a variety of cell types, leading to production of a variety of mediators that have been suggested to play important roles in lung tumorigenesis.

Our current work highlights that treatment of cells with cigarette smoke condensate for long term is a good model for studying the effect of long-term smoking in humans. On an average a person smokes for at least 50 pack years before the person actually develops lung cancer. So treating the cells for as short as 2 hours cannot correctly simulate the effects of long-term smoking. Our studies have uncovered two roles of long-term smoking in humans as summarized in Fig. 42.

In our first study we have shown that long-term CSC treatment results in reduced expression of Smad3. Smad3 expression is reduced both at the protein level and the mRNA level. Mechanistically, the expression of Smad3 is reduced due to accumulation of transcriptional repressor Sp1 on the Smad3 promoter. The treatment of histone deacetylase inhibitor, Sodium Butyrate removes Sp1 from the Smad3 promoter and thus brings back the expression of Smad3. The biological effect of reduced expression of Smad3 was increased cell viability due to reduced apoptosis. The decrease in apoptosis was due to the increased expression of anti-apoptotic Bcl2. Further, long-term CSC treatment enhanced tumorigenecity in A549 cells, partly through the down regulation of Smad3. Finally, we illustrated that in lung cancer patients the expression of Smad3 mRNA was lower in smokers compared to non-smokers.

In the second part of the study we show that the long-term CSC treatment to the cells makes the cells resistant to carboplatin. The cells become resistant due to the abrogation of induction of apoptosis by carboplatin. We further show that Bcl2 is involved in making the cells resistant to carboplatin. We also illustrated that in our in vitro model system, Smad3 acts upstream of Bcl2 and regulates the expression of Bcl2 and thus partially contributes to the resistance to carboplatin. This negative correlation between the expression of Smad3 and Bcl2 was valid in the mRNA levels too in lung cancer patients.
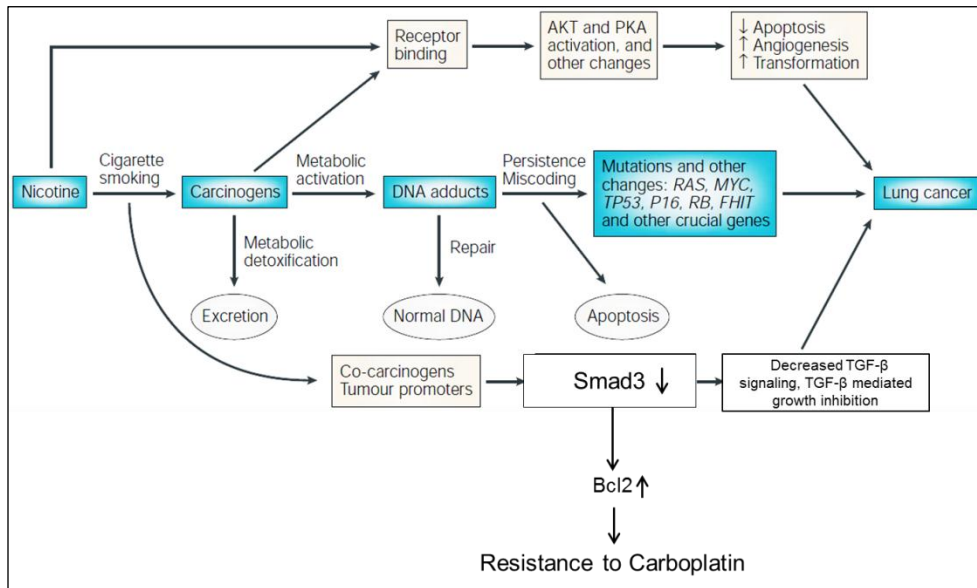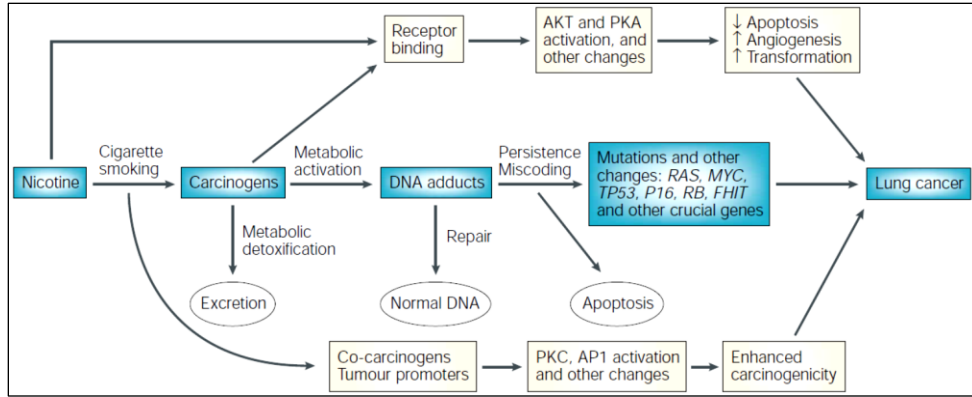
Figure 42. Summary of what the dissertation adds to the current knowledge.

The top figure shows the scheme of link between lung cancer and tobacco-smoke carcinogens. Figure first published in (Hecht 2003). Our study adds to the existing model by adding the bottom part where the treatment of co-carcinogens and tumor promoters present in cigarette-smoke condensate results in decreased expression of Smad3 and thus imparts tumorigenecity and resistance to carboplatin in lung cancer patients.

Despite the impressive progress that has been made, there are still many problems. One of them is to diagnose the smokers who are susceptible to develop lung cancer. Accurate measurement of specific carcinogen–DNA adducts in readily available samples is a significant challenge, which should be addressed as technology improves. It is also important to integrate carcinogen biomarkers into epidemiological studies. To reach this goal and obtain meaningful data, it is essential to develop methods that have adequate sensitivity and specificity for particular analytes and that are also amenable to high throughput analysis. A summary of the current molecular profiling approaches used now a days are shown in Fig.43.
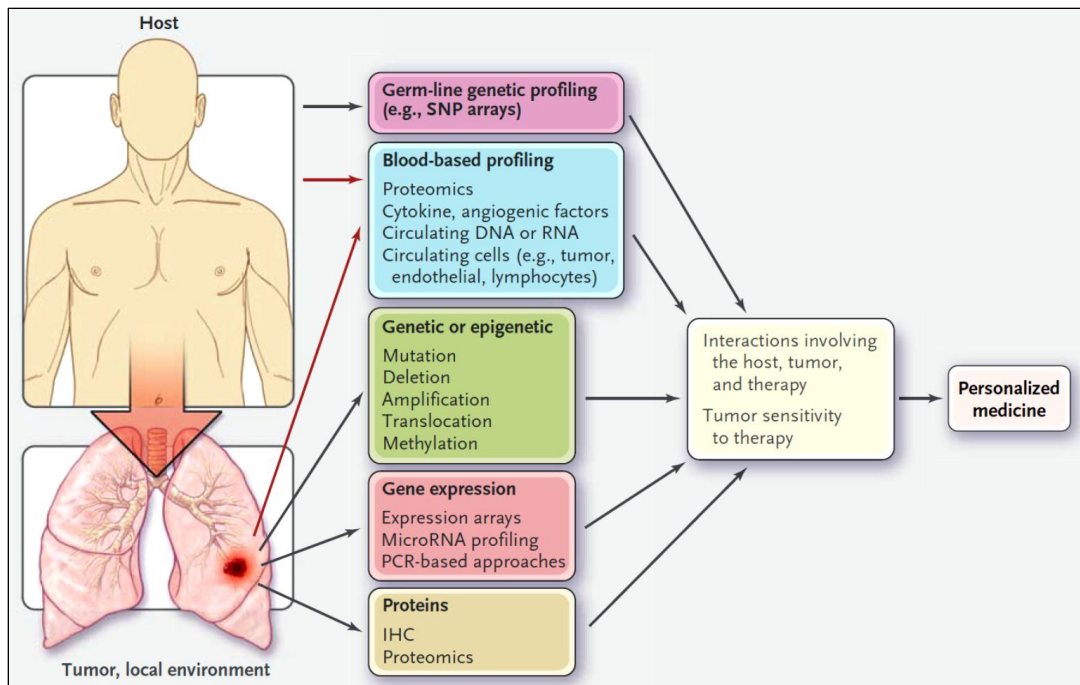
Figure 43. Molecular-Profiling Approaches to the Development of Personalized Therapy. Figure originally published in (Herbst, Heymach et. al, 2008)

Even though a lot of information have been gathered regarding the effect of tobacco carcinogens on lung tumorigenesis but still there are avenues which have not been pursued.

1. One of the most important questions might be which smoker will get cancer, given that an estimated 11-24% of smokers develop lung cancer. This question can be answered by looking at the levels of carcinogenic biomarker. The carcinogenic biomarker would help us identify the individuals who activate tobacco carcinogens more efficiently.

2. Here we have looked into how the expressions of the proteins are changed due to chronic CSC exposure. One other avenue could be to look at the mutation spectra of the genes with different time points of CSC treatment. There would be two different comparisons, one looking into the difference in the mutational spectra between 4 days, 100 days and 300 days of CSC treatment. The second comparison would be between the 4 days CSC and DMSO; 100-days CSC and DMSO and 300-day CSC and DMSO. From the mutational spectra we can get an idea about the novel if any pathways that are deregulated due to chronic CSC treatment.

3. Another avenue that can be pursued would be to look into the expression of the microRNA's due to chronic CSC treatment. Here also we can look into how different time points of CSC exposure can change the expression patterns of the microRNA's. Then we can do an unsupervised clustering and get an idea about the pathways that are deregulated due to chronic CSC exposure.

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