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Induction of synthetic lethality in mutant KRAS cells for non-small cell lung cancers chemoprevention and therapy

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Induction of synthetic lethality in mutant KRAS cells for non-small cell lung cancers chemoprevention and therapy

A DISSERTATION

Presented to the Faculty of

The University of Texas Health Science Center at Houston

and

The University of Texas MD Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment of the Requirement for the

Degree of DOCTOR OF PHILOSOPHY

By

Shaoyi Huang, M.S.

August, 2013

Houston, TX, USA

Advisor: Xiangwei Wu, Ph.D.

Dedication

To my wife and son, Xing liu and Danny Huang, and my parents, Shilong Huang and Chungui Pan, and my lovely friends and lab members, for all your love and supports!

Acknowledgements

First, I would like to thank my mentor, Dr. Xiangwei Wu. His careful guidance, inspiration and support made this dissertation possible. Thank for not giving me up when I was in the predicament. I would also like to thank my previous mentor, Dr. Yinhua Yu, who led me into the world of cancer research.

I would like to thank current and previous committee members, Drs. Carlos Caulin, Bingliang Fang, Peng Guang, Xin Lin, Walter N. Hittelman, Warren Liao, Reuben Lotan, Qiang Shen and Dihua Yu. Their advices and guidance are always the driving force for me to walk through the long journey of my Ph.D. training.

I am grateful to my current and former lab members: Dr. Jennifer S. Davis, Dr. Vineet Gupta, Dr. Yannis Hara, M.D. Xiaoyang Ren, Dr. Haizhen Wang, Dr. Xianfeng Wen, Mr. Zhengming Xu, Dr. Oksana Zagorodna and Dr. Ling Zhang, for their cooperation and help in experiments, stimulating discussions and constant support.

I would also like thank my good friends, Drs. Xiaoming Chen, Robert Luo, Teddy Lee, Ming Lu, Zhiyong Ren, Jiali Si and Xi Zhou for all the support from them.

Finally, I sincerely thank all my family members: my parents, my wife and my cute son. I want to thank you for the unconditional support and love.

Induction of synthetic lethality in mutant KRAS cells for non-small cell lung cancers chemoprevention and therapy

Publication No. _____________

By Shaoyi Huang

Advisor: Xiangwei Wu, Ph.D.

Lung cancer is the leading cause of cancer death in both men and women in the United States and worldwide. Despite improvement in treatment strategies, the 5-year survival rate of lung cancer patients remains low. Thus, effective chemoprevention and treatment approaches are sorely needed. Mutations and activation of KRAS occur frequently in tobacco users and the early stage of development of non-small cell lung cancers (NSCLC). So they are thought to be the primary driver for lung carcinogenesis. My work showed that *KRAS* mutations and activations modulated the expression of TNF-related apoptosis-inducing ligand (TRAIL) receptors by up-regulating death receptors and down-regulating decoy receptors. In addition, we showed that KRAS suppresses cellular FADD-like IL-1β-converting enzyme (FLICE)-like inhibitory protein (c-FLIP) expression through activation of ERK/MAPK-mediated activation of c-MYC which means the mutant *KRAS* cells could be specifically targeted via TRAIL induced apoptosis. The expression level of Inhibitors of Apoptosis Proteins (IAPs) in mutant *KRAS* cells is usually high which could be overcome by the second mitochondria-derived activator of caspases (Smac) mimetic. So the combination of TRAIL

and Smac mimetic induced the synthetic lethal reaction specifically in the mutant-KRAS cells but not in normal lung cells and wild-type *KRAS* lung cancer cells. Therefore, a synthetic lethal interaction among TRAIL, Smac mimetic and *KRAS* mutations could be used as an approach for chemoprevention and treatment of NSCLC with *KRAS* mutations. Further data in animal experiments showed that short-term, intermittent treatment with TRAIL and Smac mimetic induced apoptosis in mutant *KRAS* cells and reduced tumor burden in a KRAS-induced pre-malignancy model and mutant *KRAS* NSCLC xenograft models. These results show the great potential benefit of a selective therapeutic approach for the chemoprevention and treatment of NSCLC with *KRAS* mutations.

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CHAPTER 1 INTRODUCTION

1.1 Lung cancer

Lung cancer, cancer originating in the lung or bronchus, remains the leading cause of cancer death in the United States and worldwide despite over 30 years of progress in early detection and standard treatment[1]. According to the American Cancer Society estimation, lung and bronchus cancers rank the second place in new increased cases and accounts for the most death caused by cancer in 2012 for both female and male (Fig. 1)[2]. Broadly, it is divided into 2 types according to the pathology: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC) [3].

SCLC is an undifferentiated or low differentiated neoplasm composed of primary cell-like cells [3,4]. The cancer cells are usually smaller than normal cells due to mutations in the pathways controlling cell size. The mutations causing the uncontrolled cell growth majorly include retinoblastoma (RB) and P53 gene mutations [5,6,7]. Other oncogenes overexpression, like c-MET, MYC and BCL2, were also strongly associated with SCLC [5,8-10]. SCLC occurs almost exclusively in smokers and is highly aggressive due to the low differentiation grade. It is rapidly growing, and approximately 80% of patients have metastatic disease at the time of diagnosis [3,4,11]. Even though the SCLC is very responsive to chemotherapy and radiation therapy, such as platinum-containing agents, the tumors come back quickly in most patients[12]. The mechanism of drug resistance in SCLC is complicated and involved multiple factors, such as membrane protein drug pumps overexpression, enhanced cell DNA repair system and dysfunction of apoptosis system[13-15].

So SCLC is very malignant and has bad prognosis. Fortunately, it only accounts for about 15-20% lung cancer cases (Fig.2)[1,3].

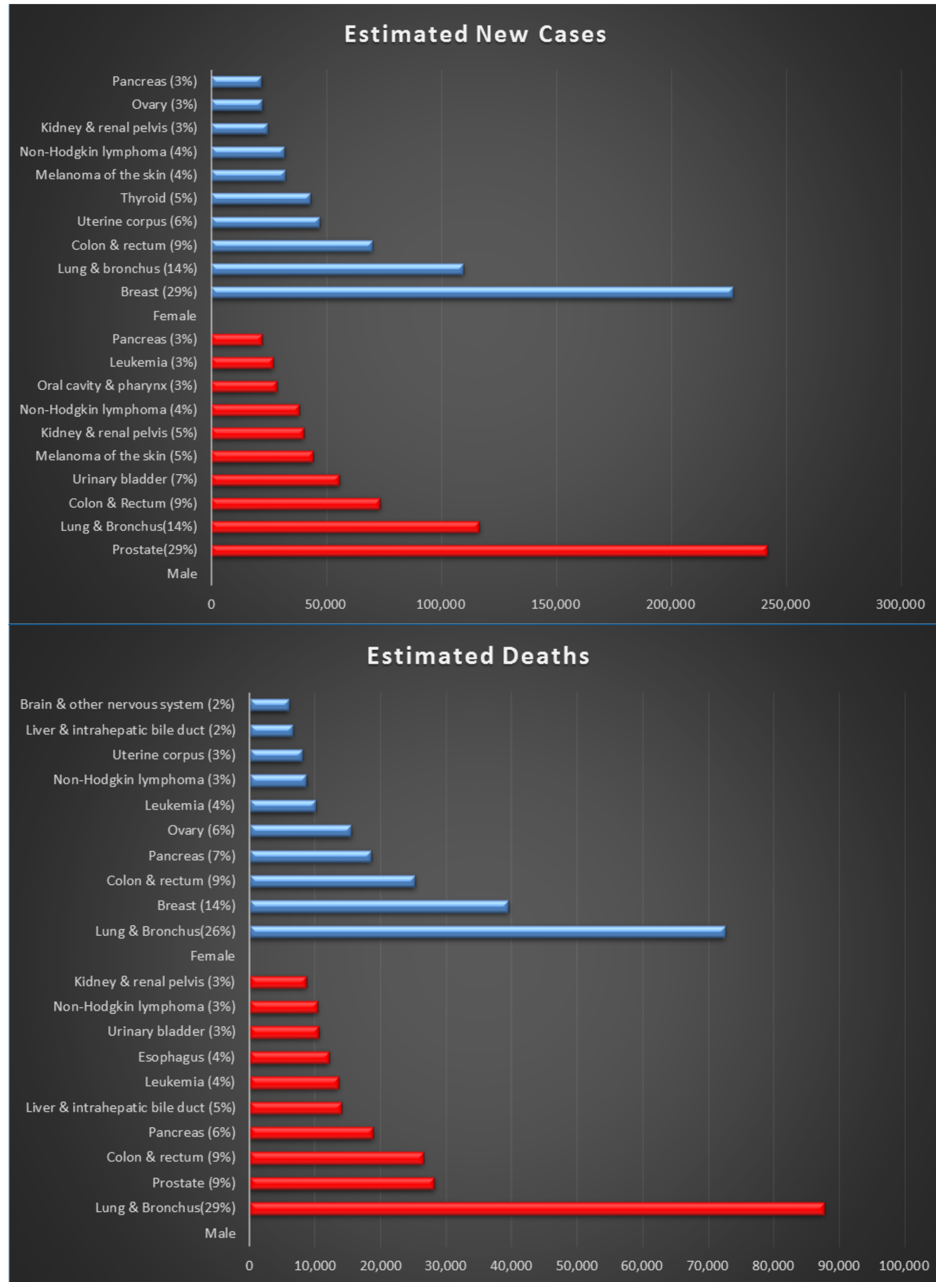

Figure 1. The estimated new cases and death caused by cancers.

(American Cancer Society, Cancer Facts & Figures 2012. Atlanta: American Cancer Society; 2012.)

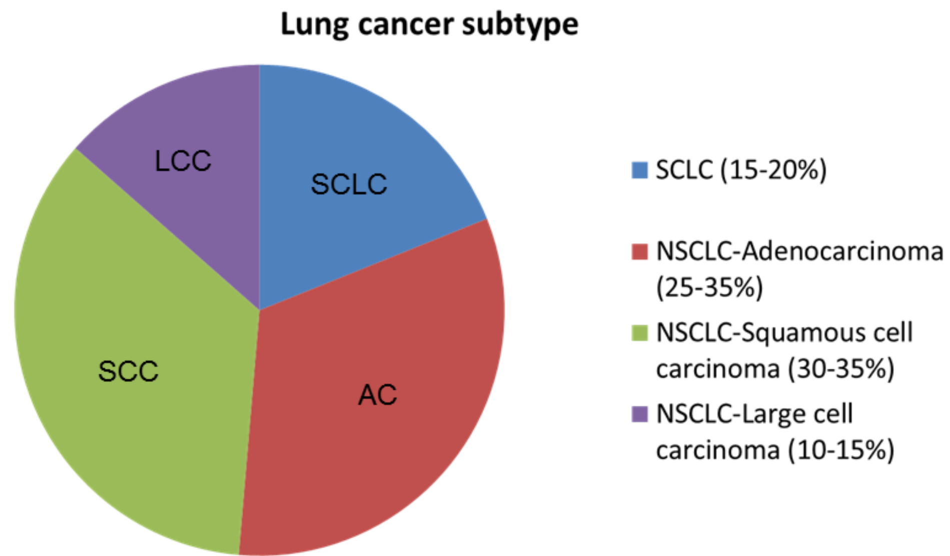

Figure 2. Subtype of lung cancer

Besides the SCLC, the most common form of lung cancer is NSCLC, which accounts for 80-85% of all lung tumors, including adenocarcinoma (25-35% of cases), squamous cell carcinoma (30–35%) and large cell carcinoma (10–15%) (Figure 2) [3,16]. The three subtypes of NSCLC are divided according to the cell type of origin, but there can also be mixed cell types in patients. NSCLC is diverse not only in the subtype, but also in the genetic background[17-20]. It usually harbors a single specific mutated oncogene that is thought to be the primary genetic "driver" gene which leads to tumorigenesis. To date, a number of driver genes have been identified including: *KRAS, BRAF, MEK1/2*, epidermal growth factor receptor (EGFR), *HER2, PI3K* and *EML4-ALK* [3,17-20]. Among those genes, mutation of *KRAS* is found in 25-30% of NSCLC[17].

1.2 RAS mutations in lung cancer

RAS gene was firstly discovered during the study of cancer-causing viruses in animals (Harvey JJ 1964, Kirsten 1967) [21-22]. Then it got the name from the rat sarcoma (Ras) and two discoverers, Harvey ras (H-Ras) and Kirsten ras (K-Ras). There was also another Ras found later in human neuroblastoma cells, so called N-Ras [23]. RAS, KRAS and NRAS all belong to the RAS family, which encode a family of membrane-bound 21-kd guanosine triphosphate (GTP)-binding proteins [24]. Those highly homologous proteins are all GTPases that act as molecular on/off switch. After binding to GTP with the help from guanine nucleotide exchange factors (GEFs), those RAS proteins will recruit and activate various downstream proteins, such as PI3K and RAF-MEK-ERK, to provide the survival and proliferation signals for the cells [24-26]. Because of the intrinsic GTPase activity of RAS, it will hydrolyze the bound GTP into GDP with the help of GTPase activating proteins (GAPs). Then the GDP-bound form is inactive for downstream signaling [24,27].

If there are any mutations in RAS which prevent the GTPase activity of RAS, the RAS will be always RAS-GTP form and constitutively active. The most common gain function mutations in RAS include residue G12, G13 and Q61 [28]. The mutations at residue 12 and 13 would attenuate the interaction of GAP to RAS and then block the hydrolysis of GTP by RAS protein, while the mutations at residue 61 directly abolish the hydrolysis activity of RAS [28-31]. Upon those mutations, RAS proteins were constitutively "on" and the downstream signaling will be activated inappropriately, which can modulate cell growth, differentiation, and apoptosis through linked receptor and non-receptor tyrosine kinases,

and finally cause the tumorigenesis [24, 29]. This is accomplished through multiple effectors including Mitogen-activated protein kinase (MAPK), Signal transducer and activator of transcription (STAT) and Phosphoinositide 3-kinase (PI3K) signaling cascades (Fig. 3) [24-26, 32]. Mutations in genes which are upstream of RAS can have similar effect as well as RAS mutation, such as BCR-ABL or EGFR [33-36].

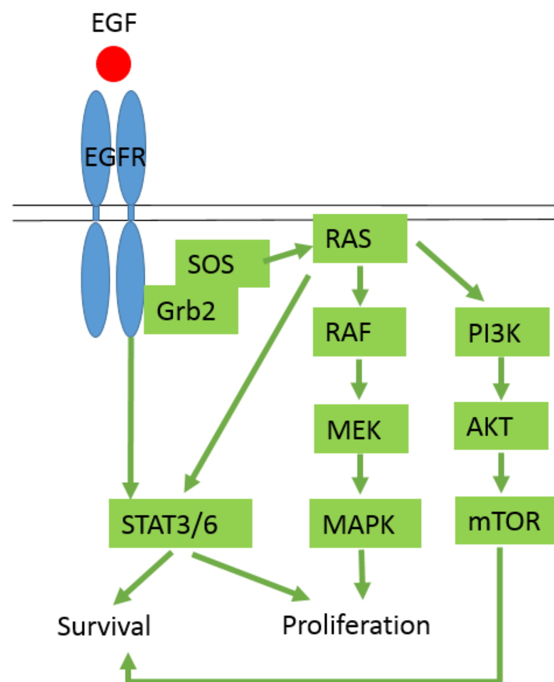

Figure 3. The RAS signaling casecades.

RAS mutations have been found in approximately 30% of all human cancers, including mutations in NRAS, HRAS and KRAS [24, 29]. Since the downstream signals of RAS genes vary by family members, there is some correlation between specific tumor type and RAS gene mutation [29, 37]. For example, in adenocarcinoma of the lung, pancreas and colon, the KRAS gene is the predominantly mutated member of the RAS family, whereas in

myeloid leukemia it is primarily NRAS that is mutated [38-41]. HRAS mutations may be related to bladder and kidney cancers [42-43].

KRAS mutations account for most of the cancer-related RAS mutations in NSCLCs [38]. The studies revealed that KRAS mutations accounts for 90% of RAS mutations in lung adenocarcinomas and approximately 97% of *KRAS* mutations in NSCLC involve codons 12 or 13, while KRAS mutations are rare in lung squamous cell cancer [44, 45]. Recent study suggests that KRAS mutations are associated with a worse overall survival in patients with NSCLC, especially in patients with adenocarcinoma and early stage [46-47]. And also some studies suggested that the lung cancers with KRAS mutations are more resistant to cytotoxic chemotherapy and EGFR-TKIs treatments [48-50]. The studies in recent two decades did not provide any effective way to treat the NSCLC with KRAS mutations.

1.3 Lung cancer treatment

Symptoms of lung cancer include cough, chest pain and weight loss. However, many patients present with metastatic cancer having no obvious clinical symptoms. Diagnosis is typically made by chest x-ray or CT and confirmed by biopsy. Most NSCLC patients are detected when the cancer is already advanced [51]. Depending on the stage of the disease, treatment may include surgery, chemotherapy, radiation therapy, or a combination. For the past several decades, the prognosis for a lung cancer patient was poor, with only 15% of patients surviving > 5 year from the time of diagnosis [52]. For patients with stage IV (metastatic) disease, the 5-year overall survival rate was < 1% [52].

However, the identification of certain mutations that can be targeted for therapy, has recently improved outcomes.

Targeted therapy has been established for the treatment of advanced NSCLC, such as treatment with erlotinib (Tarceva) after chemotherapy, which targets the *EGFR* mutation. But since KRAS is downstream effector of EGFR, the erlotinib has little benefit for those NSCLC patients with *KRAS* mutation [48-50]. In recent years, several new targeted therapies have been developed for *KRAS* mutation, such as the MEK1/2 inhibitor AZD6244 (Selumetinib) [53], the BRAF inhibitor Sorafenib (Nexavar; BAY 43-9006) [54] and the Farnesyl Transferase Inhibitors (FTIs) [55-56].

The MEK1/2 inhibitor and BRAF inhibitor function via directly binding to the MEK1/2 and BRAF individually and inhibiting the Kinase activity. The study on the MEK1/2 and BRAF inhibitors showed that the combination of Selumetinib plus docetaxel as the second-line treatment of KRAS mutated NSCLC significantly improved outcomes. The primary end point of overall survival was increased from 5.2 months to 9.4 months [57]. A Phase II clinical trial of Sorafenib for the treatment of NSCLC in 2012 also showed some efficacy [58]. The primary end point of overall survival from time of randomization was 13.7 months in the treated group versus 9.0 months in placebo group [58].

The FTIs can bind to farnesyl transferase (FFTase) and inhibit the farnesylation of KRAS on the C-terminal of protein. The post-transcription modification of KRAS, including farnesylation, methylation and palmitylation, will associate KRAS protein to inner face of the plasma membrane, which is essential for RAS interaction with other proteins [59].

Several Farnesyl Transferase inhibitors had been tested in clinical trials, including lonafarnib (SCH-66336), R115777 and L-778,123 [60-62]. Even those inhibitors showed the anti-cancer activity in pre-clinical experiments and some phase I/II studies, there was no improvement in overall survival in phase II/III studies [56]. The failure of FTIs may be due to the alterations of farnesylation of KRAS, such as myristylation and geranylgeranylation, which will also associate the KRAS protein to the memberane [63].

These outcomes for KRAS targeted therapy are promising but not very impressive. In contrast, treatment with crizotinib led to an overall survival of about 24 months in patients with an (anaplastic lymphoma kinase) ALK rearrangement, while treatment with erlotinib increased overall survival to about 27 months in patients with an *EGFR* mutation [64-65]. The KRAS targeting drugs also had some side effects, which cause decreased dose or interrupted dose [66]. Toxicity concerns, together with the relatively modest increase in survival of current KRAS targeted therapies, highlight the urgent need to develop new strategies or drugs targeting *KRAS* mutant NSCLC.

1.4 Lung cancer chemoprevention

KRAS mutations are not only found in cancers but also in individuals at risk (but without cancer) who have significant tobacco exposure and are detected in 25%–40% of atypical adenomatous hyperplasia lesions, which are a potential precursor of adenocarcinoma [67]. This suggests that *KRAS* mutations exist not only in advanced NSCLC, but also appear to be an early event in human NSCLC development, and may be regarded

as the driving event of NSCLC [67-70]. So KRAS mutations could be the good target for cancer chemoprevention.

Cancer chemoprevention is the use of synthesized drugs or natural compounds to suppress or slow down the carcinogenesis process or prevent the recurrence of a tumor after successful treatment [71]. Cancer chemoprevention represents an important facet of cancer research because cancer, especially late-stage cancer, remains the second killer in U.S.A and worldwide [72]. Many years of research has led to great improvement in our understanding of the molecular mechanisms of cancer and to the development of various advanced treatment procedures. However, progress has been slow in the long-term survival rate for patients with most solid tumors. The difficulty in treating late-stage cancer results from its intrinsic property: genomic instability. The current chemotherapy and radiotherapy may not kill 100% of the cancer cells and the remaining cells exhibit poor drug response, which is in part responsible for the high relapse rates in cancer treatment. Cancer is a multi-step disease, the development of which requires multiple genetic and epigenetic changes [3, 73]. Chemoprevention targets tumor development at early stages, including tumor initiation and promotion when the cancer genome remains relatively stable. Since the most difficult obstacle in cancer treatment is genomic instability, bypassing it using this approach holds great potential.

However, only a few molecular targets have been identified and the molecular basis for chemoprevention is poorly understood. To date only about 13 agents are FDA approved for cancer chemoprevention. They include Tamoxifen, Raloxifene, HPV vaccine,

Celecoxib and Fluorouracil [74]. Tamoxifen and Raloxifene are approved to reduce the risk of breast cancer incidence in high-risk women [75-76]. The HPV vaccine is approved for the prevention of cervical and anal cancers caused by HPVs [77]. Celexcoxib is a non-steroidal anti-inflammatory drug (NSAID) and specific COX-2 inhibitor which has been shown to decrease the number of adenomatous colorectal polyps in FAP patients as an adjuvant to regular care (such as endoscopic surveillance and surgery) [78]. Fluorouracil (5Fu) is used as a chemotherapy drug to treat several types of cancer including colon, rectum, and head and neck cancers. It was recently approved by the FDA for topical treatment of actinic keratosis (AKs) [79]. Untreated AKs can become squamous cell carcinomas (SCC), the second most common form of skin cancer. In addition to these approved drugs, more agents are being investigated in labs for use in chemoprevention [74, 80-82]. Phytochemicals, including curcurmin and genistein, represent a popular class of potential chemoprevention agents [83-84]. Based on epidemiological studies, it has been demonstrated that these compounds have anti-inflammatory activities and can reduce the risk of carcinogenesis [85].

Despite the handful of FDA approved chemoprevention drugs, there are still a lot of difficulties in current chemoprevention strategies. For those phytochemicals, the mechanism of anti-inflammatory activity remains unclear. Without a precise assessment of the mechanisms, it is unlikely that phytochemicals will be recommended for testing in human clinical trials. Even for those approved chemoprevention drugs; successful treatment requires long-term administration, which can lead to toxicity, high cost and resistance. For lung cancer, some agents, such as COX2 inhibitors, have shown activity against lung cancer in animal [86]. However, in clinical trials the effect is not very obvious and

long-term continuous administration is required, which causes safety concerns [87]. Even the approved chemoprevention drugs, Tamoxifen and Raloxifene, achieved 50-70% reduction in incidence of invasive breast cancer in the treatment arm, toxicity and resistance are still notable concerns [74, 88]. In an effort to overcome these challenges, we need to develop new chemoprevention approaches that can specifically target premalignant tumor cells or malignant tumors.

In the lung cancer field, there are also some drugs and methods being studied specifically targeting chemoprevention. Several natural products have been tested in clinical trials for lung cancer chemoprevention in smokers, including beta-carotene, retinol, Vitamin E or N-acetylcysteine [89-93]. Unfortunately, these trials showed either neutral or harmful primary endpoint results in primary, secondary or tertiary prevention [89-93]. Trials of aspirin and selenium also had little to no positive effect [94-95]. Recently, there have been a number of studies focused on NASIDs for lung cancer chemoprevention, particularly the COX-2 inhibitors [96-97]. This is because inflammation has been correlated with carcinogenesis and COX-2 over-expression has also been observed in former smokers. Additionally, COX-2 over-expression is shown to be a poor prognostic indicator in NSCLC [98-99]. The most well-known COX-2 inhibitor is celecoxib, which inhibits the production of PGE2 and reduced the Ki-67 labeling index in active smokers in a phase II clinical trials [100]. However, the large scale chemoprevention trials of celecoxib had only neutral results [96-97]. There is still no clear path for the development of lung cancer specific chemoprevention strategies.

1.5 SITEP based cancer chemoprevention

For most traditional approaches of chemoprevention, it usually applies the agents to modulate the tumorigenic pathways rather than eliminate large numbers of premalignant or malignant cells, which will also affect the normal cells [101]. To keep the toxicity to the minimum or the lowest tolerated level of patients, only very limited dose of agents had been applied for chemoprevention, which will lead to low efficacy. To maintain the efficacy of chemoprevention, long term continuous or near continuous treatment could not be avoided, which will cause the accumulated toxicity and bring some unexpected side effects.

In contrast to those traditional long term and continuous dosing treatment for chemoprevention, a new idea called short-term intermittent therapy to eliminate premalignancy (SITEP) has been raised based on the synthetic lethality strategy [102]. The core goal of SITEP is to reduce the long-term toxicity, while maximizing efficacy, by using short term and intermittent treatment to prevent cancer. This goal can be achieved because of the nature of carcinogenesis. Carcinogenesis is a multistep process that involves the accumulation of many genetic and epigenetic changes [3, 73, 89]. Typically only a few crucial mutations will drive normal cells to become premalignant cells and finally malignant cells. The SITEP approach is to kill the premalignant cells by targeting the crucial mutations via short term and intermittent treatment. Intermittent treatment can reduce the number of premalignant cells and ultimately decrease the patient's risk of developing cancer between treatment cycles (Fig 4).

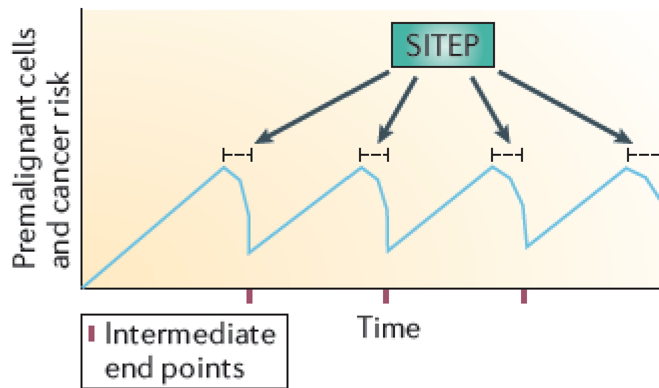

Figure 4. The principle of SITEP. The number of premalignant tumor cells is positively proportional to the cancer risk in patients. Intermittently eliminating premalignant tumor cells with short time treatment should dramatically decrease the cancer risk and subsequently inhibit or delay cancer development.

(Xiangwei Wu and Scott M. Lippman, *Nature Reviews:Cancer*, 2011, vol 11)

1.6 Synthetic lethality

One of the key factors for SITEP is to eliminate a large numbers of premalignant cells by a short term treatments. That requires potent and selective killing effects on the premalignant cells in a short time. Synthetic lethality might be an appropriate strategy for that purpose.

Synthetic lethality means the cell death could be induced by the combination of mutations in two or more sensitive genes, while mutation of single gene alone is not sufficient to induce cell death [102]. Compared to the current cytotoxic drugs, the synthetic lethality approach has the advantage of inducing apoptosis selectively and precisely in those mutated cells while not harming the normal cells. Compared to the current targeted therapy drugs, such as the kinase inhibitors or antagonists, synthetic lethality has the

advantage of changing the targeting effect from cytostatic to cell death, which is more efficient and less toxic. Based on the idea of synthetic lethality, it is recently reported that apoptosis could be induced specifically in the premalignant cells with particular gene mutations, BRCA1/2 mutation [103]. Poly (ADP-ribose) polymerase (PARP) inhibitors hinder base-excision repair and cause cell death in BRCA1/2 deficient cells due to DNA damage and could be used to target the BRCA1/2 mutant breast premalignant cells. Our lab also reported that Retinoic Acid (RAc) plus TRAIL, which targets the APC deficient colorectal premalignant cells, have the potential to be effective chemopreventive agents [104]. RAc plus TRAIL induces apoptosis only in the APC deficient cells due to the up-regulated cMyc level, resulting in reduced cFLIP levels, will sensitize the cells to TRAIL triggered extrinsic apoptosis. The outcome of animal experiments with APC/Min mice has proved the great potential for chemoprevention.

1.7 Apoptotic pathway and TRAIL

Apoptosis is one type of Programmed Cell Death (PCD) which exists in difference organisms [105]. It is usually characterized by distinct morphological changes, energy-dependent biochemical cascades and finally cell [105-106]. The morphological changes include membrane blebbing, cell shrinking, chromatin condensation,
and chromosomal DNA fragmentation [105-106]. The intrinsic biochemical processes include activation of caspase cascades, protein cleavage, protein cross-linking and DNA breakdown [105-107].

Caspases are a family of proteases which are able to cleave proteins at aspartic acid residues [108]. So far about 10 caspases have been identified and categorized into 3 groups. They are initiators (caspase-2,-8,-9,-10), effectors or executioners (caspase-3,-6,-7) and inflammatory caspases (caspase-1,-4,-5) (14). Pro-caspases can be activated through either intrinsic or extrinsic apoptotic pathways by cleavage [108-109]. The intrinsic apoptotic pathway activates caspases via permeabilization of the mitochondria and release of pro-apoptotic proteins into the cytoplasm, such as cytochrome c, Apaf-1 and second mitochondria-derived activator of caspase (Smac) [110-112]. Cytochrome c and Apaf-1 form a multi-protein complex known as the "apoptosome" and initiate activation of the caspase cascade through cleavage of caspase-9 [111]. The extrinsic apoptotic pathway activates the caspases via transmembrane death receptors such as tumour necrosis factor receptor 1 (TNFR1), Fas/CD95 and TRAIL receptors (DR4/5) [112-113]. As death ligands bind to these receptors, the death inducing signaling complex (DISC) is formed leading to initiation of the caspase cascade through cleavage of caspase-8/10 [114]. Once caspase-8/10 is activated, the downstream caspases are triggered, and the cell undergoes apoptosis [112-114].

Researchers observed apoptotic cells in different cancers and found that a high rate of apoptosis is correlated with slow growing tumors, and increased apoptosis was also observed in tumors treated with radiation or chemotherapy [115]. Elsewhere, researchers reported that failure of apoptotic activation may promote cancer growth and even cause [116-117]. This may be the case in tumors with Bcl-2 overexpression not only survives the removal of growth factors, but is also resistant to chemotherapy [116]. Taken together, this indicates that induction of apoptosis may be an interesting approach for

cancer treatment. Agents that can restore the apoptotic signaling pathways may specifically kill the cancer cells, which require these apoptotic defects to survive. A lot of studies have focused on these agents, which has opened the door into a new type of anticancer drugs and treatment strategies [118-120]. This class of drugs includes Bcl-2/Bcl-xL inhibitors, Bcl-2/Bcl-xL siRNA, XIAP siRNA and caspase activating drugs. Among them, the death receptor ligand tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a popular candidate and has great potential [116].

TRAIL was first cloned in the 1996 [121] and following studies showed that TRAIL forms a homotrimer and binds to its death receptors 4 and 5 (DR4, DR5) on the cell surface [122]. Both DR4 and DR5 have 2 extracellular cysteine-rich domains and one cytoplasmic death domain (DD) [122]. Upon binding to TRAIL, DR4 and DR5 also form homotrimers or heterotrimers and recruit Fas-associated death domain (FADD) through the DD interaction [123-124]. Then FADD recruits amino terminal death effector domain (DED)-containing apoptosis initiating proteases, caspase-8/10 via its own DED domain [123-124]. The cytoplasmic DD domain of DR4/5, the FADD and the caspase 8/10 form a death-inducing signaling complex (DISC) [123-124].The formation of DISC will cause caspase-8/10 auto-cleavage and active, which will trigger the downstream process including cleavage of downstream effector caspase-3/7 and DNA fragmentation [125]. In addition to DR4 and 5, three decoy receptors of TRAIL, DcR1, DcR2 and OPG, can also interact with TRAIL. These decoy receptors may exist in both membrane-bound and soluble form [126]. When bound to the membrane, the lack of functional cytoplasmic signaling domains will block the TRAIL induced death signaling [126]. Upon TRAIL binding to DR4 or DR5, death receptor mediated

apoptosis can be inhibited by a protein called cellular FADD-like IL-1β-converting enzyme (FLICE)-like inhibitory protein (c-FLIP), which binds to FADD and pro-caspase 8/10 and prevents the cleavage of pro-casepase 8/10 (Fig.5) [127].

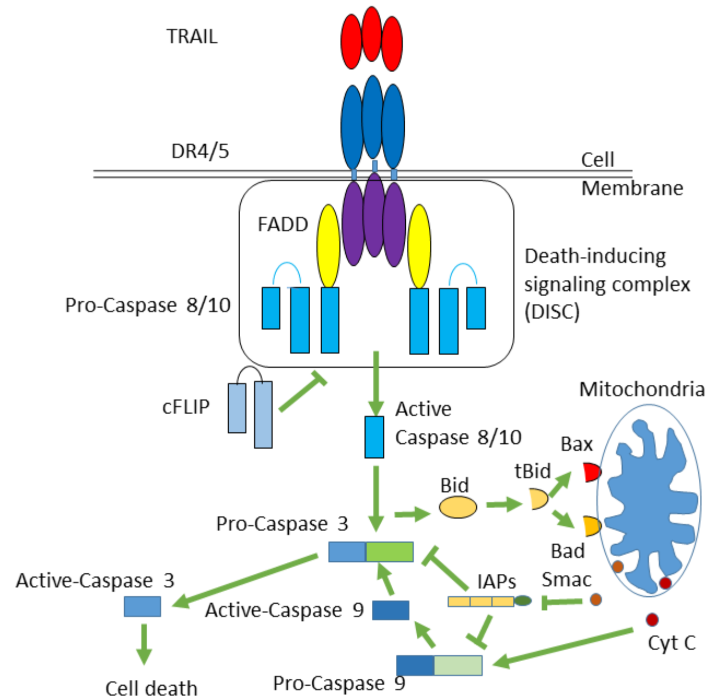

Figure 5. The intrinsic and extrinsic apoptotic pathways of TRAIL

In recent years, TRAIL has shown the great potential as an anti-tumor candidate by selectively killing a wide variety of human tumor cell lines without harming normal cells [125]. This discovery resulted in the rapid development of cancer therapeutics targeting this pathway. The TRAIL pathway has been targeted for clinical application by at least two approaches: recombinant human TRAIL (rhTRAIL) ligand and agonistic antibodies against DR4 and DR5 [126-129]. The toxicity of rhTRAIL and the DR4/DR5 monoclonal antibodies (Mapatumumab and Apomab) has been evaluated in a variety of animal assays, with little

or no toxicity [128-129]. Both rhTRAIL and TRAIL agonists have been moved to in phase II clinical trials [125]. TRAIL showing low toxicity to normal cells usually depends on the overexpression of decoy receptors to prevent TRAIL–induced apoptosis by either binding competition or another mechanism [130]. Tumor cells frequently develop strategies to resist TRAIL induced apoptosis, including overexpression of decoy receptor, cFLIP and Inhibitors of Apoptosis Protein (IAPs) [131-134].

1.8 IAPs and Smac

IAPs belong to a family of proteins which inhibit caspase activation by binding to them and preventing cleavage of their substrates. All IAPs consist of one or more baculoviral IAP repeat (BIR) domains while some IAPs also contain a RING domain which acts as an E3 ubiquitin ligase. X-linked inhibitor of apoptosis (XIAP) and cIAP1/2 are examples of RING domain containing IAPs. The most well characterized mammalian IAP is XIAP, which can bind to and inhibit caspases-3, -7 and -9 via its BIR domains (Fig. 6) [135].

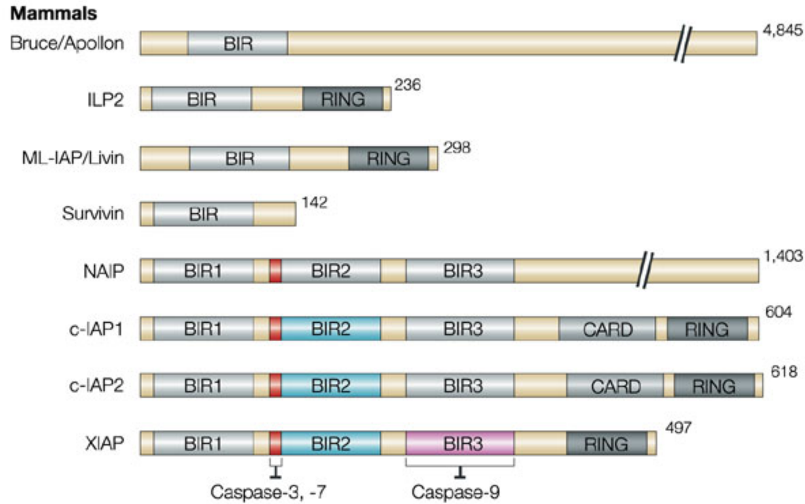

Figure 6. The family of IAPs in mammalian cells

(Stefan J. Riedl and Yigong Shi, *Nature Reviews:Molecular Cell Biology*, 2004, Vol 5)

To overcome the blockade of IAPs, TRAIL-induced extrinsic apoptotic pathway therefore requires help from the intrinsic mitochondrial pathway via caspase-8 cleavage of BH3 interacting domain death agonist (Bid) [136]. Truncated Bid (tBid) can interact with Bax and Bak and induces the oligomerization of Bax and Bak in the mitochondrial membrane, which leads to activation of the intrinsic apoptotic pathway and release of Smac from the mitochondria [136]. Smac subsequently binds to the BIR domain of the IAPs, relieving the blockade and leading to apoptosis [137-138].

Smac is a 25-kDa protein which is expressed in mitochondria and is released during the apoptosis [139]. Experiments using recombinant proteins have shown that caspase inhibition by IAPs can be relieved by the addition of IAP antagonist *in vitro* [140.] Hence the molecules that mimic the binding interactions between IAPs and Smac, referred to as Smac

mimetics, are being designed as a novel class of anticancer drugs through targeting IAP proteins, and thus sensitizing cells to induction of apoptosis. So far there have been numerous reports showing that different Smac mimetics have strong anticancer activities [141-145]. Several Smac mimetics are being tested in clinical trial, such as TL32711 in phase I study of adult patients with advanced solid tumors and lymphoma [141]. For this drug, no dose-limiting toxicities have been observed and there is strong evidence of anti-tumor activity. Other Smac mimetics being tested include LCL161 (Novartis) [142], GDC-0917 (Genentech) [143], HGS1029 (Human Genome Sciences) [144] and AT-406 (Ascenta) [145]. Our lab received Smac mimetics JP1584 from Joyant Pharmaceuticals (Dallas, TX) as a gift. The JP1584 is a small molecule and the secondary generation of Smac mimetics developed by Joyant Pharmaceuticals. It is derived from the first generation of Smac mimetics JP1010, which is a synthesized dimeric peptide (Fig. 7)[146].

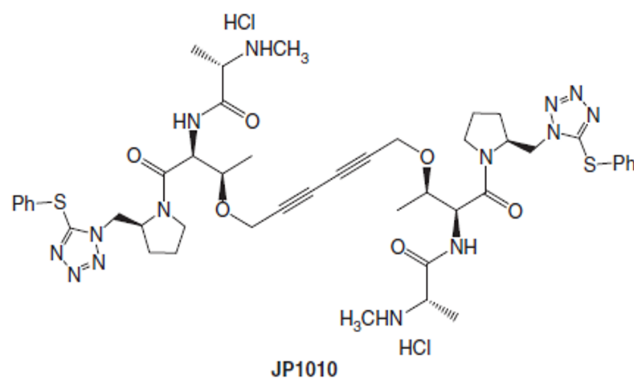

Figure 7. The Chemical structures of JP1010.

1.9 Statement of problem, hypothesis and project goals

Lung cancer is the leading cause of cancer death in both men and women in the United States. Despite improvement in treatment strategies, the 5-year survival rate of lung cancer patients remains low. Thus, effective chemoprevention and treatment approaches are sorely needed.

Recently our lab published a paper in Nature, which reported a synthetic lethal interaction between TRAIL, RAc and the APC gene (Fig. 5) [104]. We showed that TRAIL and RAc cooperatively induced apoptosis in APC-deficient cells without harming normal cells. Furthermore, short-term treatment with TRAIL plus RAc significantly reduced polyp numbers by up to 90% in APC-deficient mice. These results suggest that the combination of TRAIL and RAc have great potential in eliminating premalignant tumor cells and preventing tumor-related death in these animals.

The APC-deficiency mediated cMyc up-regulation is the key factor that sensitizes the cells to TRAIL plus RAc combination treatment. It was also reported that KRAS activation can up-regulate cMyc [147]. Considering the fact that mutations and activation of KRAS occurs frequently in NSCLC and these changes are thought to be primary drivers for lung carcinogenesis, I hypothesized that the premalignant lung cells and lung cancer cells with KRAS mutations could be specifically targeted for TRAIL induced apoptosis (Fig.8).

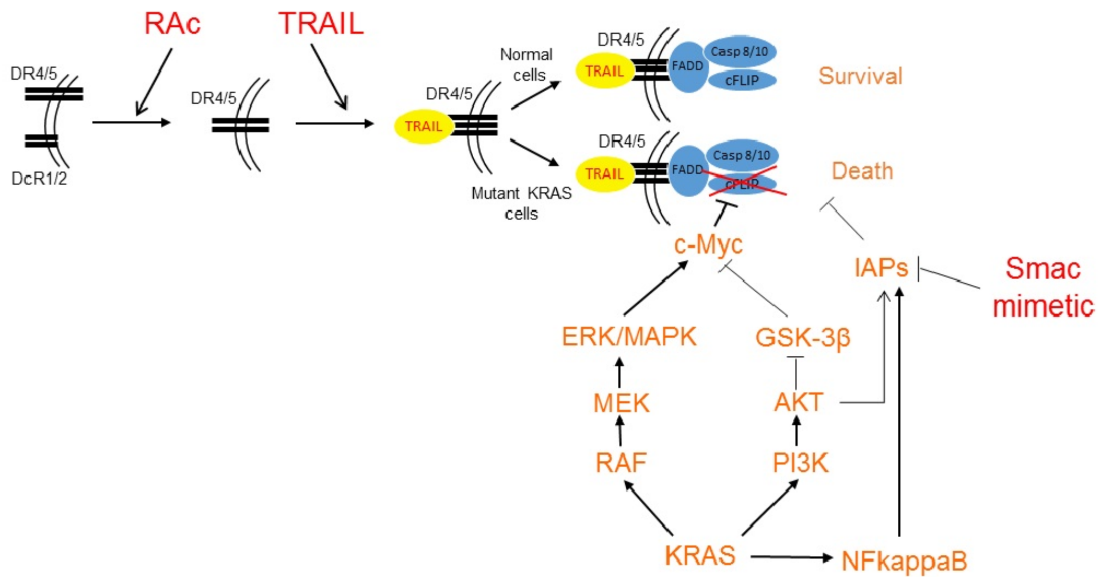

Figure 8. Synthetic lethal interaction between TRAIL, RAc, Smac mimetic and KRAS

The Inhibitors of Apoptosis Proteins (IAPs) in those cells with abnormal KRAS activation are usually up-regulated to antagonize apoptosis. The blockade can be overcome by the Smac mimetics, as reported in the literature [132]. Since KRAS mutation is also preserved in malignant stages, it is reasonable to propose that combination treatment could also be efficacious in the treatment of cancer.

Pursuing a new approach to specifically kill premalignant cancer cells would enable us to develop a more effective strategy for cancer prevention. In this way, we could make chemoprevention work like therapy to reduce the duration of treatment. The SITEP approach, applying synthetic lethality to target KRAS dys-regulation, will also minimize the potential side effects and reduce the costs associated with long-term therapy.

Given this information, we hypothesize that the co-treatment of TRAIL, RAc and Smac mimetic will induce a synthetic lethal interaction specifically in the mutant-KRAS cells, and that this approach may be applied as a new method to prevent and treat NSCLC with KRAS mutation.

CHAPTER 2 MATERIALS AND METHODS

2.1 Cell culture

The immortalized human bronchial epithelial (HBE) cell line HBE4-E6/E7 (HBE4) was purchased from ATCC. BW1799 cells were kindly provided by Dr. Reuben Lotan of M.D. Anderson Cancer Center and were maintained in Keratinocyte-SFM medium (Life Technology, Grand Island, NY). Lung cancer cell lines NCI-H322, NCI-H661, NCI-H460, NCI-H358, NCI-H157, A549, NCI-H2122 and NCI-H1299 were all purchased from ATCC and maintained in RPMI 1640 medium (Thermo Scientific , Rockford, IL) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotics (penicillin/streptomycin).

2.2 Plasmids, shRNAs, and reagents

The retroviral plasmid expressing a mutant KRAS (KRASV12) was reported in previous paper [156] and generously provided by Dr. Jinsong Liu from UT M.D. Anderson Cancer Center. ERK dominant-negative and AKT/PKB dominant-negative constructs were generously provided by Dr. Mien-Chie Hung from UT M.D. Anderson Cancer Center. The long form c-FLIP construct was generated in pcDNA3.1 (Life Technology, Grand Island, NY) by cloning cellular c-FLIP cDNA from total RNA of Hela cells, as previously reported [136].The DR5 expression plasmid was provided by W. El-Deiry. The c-Myc-shRNA was generated in pSUPER and the target sequence is 5'-TTCAAGAGA-3' [104]. XIAP-shRNA was purchased from Open Biosystems. Recombinant soluble rhTRAIL protein was purified according to

published methods[157]. In the purification, the B-PER buffer and Ni-NTA agarose were purchased from Thermo Scientific [146]. The Fluorouracil (5Fu) was purchased from Sigma (St. Louis, MO). U0126 and Wortmannin were purchased from CalBiochem (Billerica, MA).

2.3 Antibodies

Anti–c-FLIP monoclonal antibody was purchased from ALEXIS Biochemicals (Farmingdale, NY). Anti–phospho-ERK, anti–phospho-AKT, anti–cleaved CASPASE 3, anti-CASPASE 8 and anti-DR5 antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti–c-MYC and Anti-DR4 antibody was purchased from Millipore (Billerica, MA). Anti–β-Actin and anti–α-TUBULIN antibodies were purchased from Sigma (St. Louis, MO). Antibodies against DCR1 and DCR2 were purchased from Imgenex (San Diego, CA). Anti-XIAP, anti-BCL2 and anti-BCL-XL antibodies were purchased from BD Transduction Laboratories (San Jose, CA).

2.4 Infection, transfection, and drug treatment

Retrovirus was generated by using the BOSC23 packaging cell line, and infection was carried out as reported previously [158]. Transfections were carried out by using Lipofectamine 2000 (Life Technology, Grand Island, NY) according to the manufacturer's instructions. For TRAIL and Smac mimetic treatment, cells growing in log phase were treated with Smac mimetic at a final concentration of 100nmol/L for 30 minutes. Then

TRAIL was added to the media at a final concentration of 100ng/mL. For 5-Fu treatment, cells growing in log phase were treated with 5-Fu at a final concentration of 5ug/mL for 12-16 hours. Cells were harvested after 24 hours of TRAIL treatment. Where indicated, cells were treated with Wortmannin (200 nmol/L) and U0126 (10 mmol/L) for 30 minutes. Cell viability was determined by using Annexin V-FITC Apoptosis Detection Kit (Sigma, St. Louis, MO) according to the manufacturer's instructions.

2.5 Mouse Model for Tumorigenesis

Breeding colonies were established from LSL-$Kras^{G12D}$ mice acquired from the Mouse Repository of the National Cancer Institute. AdenoCre virus was purchased from the Gene Therapy Core of Baylor College of Medicine. Mice were infected according to a previously reported protocol [159-160]. In brief, AdenoCre-calcium phosphate (AdCre-CaPi) precipitates were prepared by placing recombinant adenovirus in 1 mL of Eagle's minimal essential media containing 1.8 mmol/L Ca^{2+} and 0.86 mmol/L Pi. Then an aliquot of a 2 mol/L $CaCl_2$ solution was added to achieve a concentration of 4.5 mmol/L Ca^{2+}. The solution was mixed by vortex or gentle pipetting, and the mixture was allowed to incubate for 20 to 30 minutes at room temperature. G12D mice were anesthetized with avertin at 8-12 weeks of age. AdCre-CaPi coprecipitates [$5 - 10^8$ pfu (plaque forming units)] were administered intranasally in two 62.5-µL instillations. The second instillation was administered when breathing rates had returned to normal following the first administration. Six weeks after infection, the mice were injected intravenously with Smac

mimetic (3 mg/kg). Six hours later, TRAIL (3 mg/kg) was administerd by intraperitoneal (i.p.) injections. This regimen was repeated 18 hours later. Consecutive injections of TRAIL and Smac mimetic were given a total of 3 times. Injection of PBS was used as a control. For intermittent treatment, the mice were given 3 consecutive injections of TRAIL and Smac mimetic (1.5 mg/kg) within a week, left off treatment for 3 weeks, followed by another week of injections, for a total of 3 treatment cycles. The mice were sacrificed 1 day after the last treatment, and their lungs were inflated with formalin and fixed in formalin overnight.

For the xenograft model, male athymic nude mice aged 2 months were purchased from Jackson Labs. The nude mice were inoculated subcutaneously at the right flank with 1×10^6 NCI-H322, or NCI-H460 cells in 100 µl of PBS [161]. Treatment was started once the solid tumor reached 5 mm, mean diameter (about 12 days after NCI-H322 or NCI-H460 incubation). In treated group, 3 mice were injected intravenously with Smac mimetic (3 mg/kg). Six hours later, TRAIL (6 mg/kg) was injected i.p.. Injections were repeated 18 hours later. Consecutive injections of TRAIL and Smac mimetic were given every another day for a total of 6 treatments. To minimize measurement variability, tumors were measured every 3 days, by a single individual using the same calipers. Tumor volume was calculated using the following formula: $V (mm^3) = [width^2 (mm) \times length (mm) \times \pi]/6$. On day 24, mice were sacrificed and tumors were fixed in formalin overnight. All animal experiments were conducted according to the ethical standards of the U.T.MD Anderson Cancer Center Animal Care and Use Committee (ACUC).

2.6 HE and IHC staining

Lung sections were analyzed by hematoxylin and eosin (HE) and immunohistochemistry (IHC) staining using an anti–cleaved caspase 3 antibody. Tissue sections were de-paraffinized and rehydrated in an ethanol series. For HE staining, sections were stained in Mayer's hematoxylin solution (Sigma, St. Louis, MO) for 2 min, then rinsed in 0.1% HCl-ethanol solution for 2 sec. The differentiation will take about 5 min in running tap water. Then the sections were stained in eosin Y solution 0.1% aqueous (Sigma, St. Louis, MO) for 3-5 min and rinsed in tap water for 30-45 sec. After checking the staining quality under the microscope, the sections were dehydrated again and mounted with mounting medium (Vector labs, Burlingame, CA). For IHC staining, the sections were blocked for non-specific binding with 5% goat serum and incubated with the primary anti-cleaved caspase 3 antibody for overnight at 4°C. On the second day, the sections were incubated with biotinylated secondary antibody for 1 hour at 37°C and then Avidin/Biotinylated HRP complex (Vector labs, Burlingame, CA) for 1 hour at 37°C. The DAB development were operated with the DAB Kit (Vector labs, Burlingame, CA) according to the manufactory instructions.

2.7 TUNEL assay

Apoptosis in tumor sections was analyzed using Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining with the kit from Sigma (St. Louis, MO). Tissue sections were de-paraffinized and rehydrated in an ethanol series. Pre-treat the

sections with proteinase K solution from the kit and incubate the sections with TdT reaction mixture for 1 hour at 37°C. Stop the reaction with stop solution and rinse the sections with PBS. Finally the sections were counterstained with 4’,6’-diamidinio-2-phenylindole (DAPI) and mounted with Vectashield hard set mounting medium (Vector labs, Burlingame, CA).

2.8 Statistical analysis

We compared differences between groups via 1-way ANOVA. Values with a $P < 0.05$ were considered to be statistically significant.

CHAPTER 3 Use TRAIL and Smac mimetic for lung cancer chemoprevention

KRAS mutations are found in individuals who have significant tobacco exposure and also detected in 25-40% atypical adenomatous hyperplasia lesions, which suggests that *KRAS* mutations is an early event in human NSCLC development. And also it is reported that KRAS is one of the most important driver genes in NSCLC tumorigenesis [19]. So we targeted KRAS mutations for NSCLC chemoprevention. In this chapter, first I tested the response of normal lung epithelial cells with KRAS activation to TRAIL and Smac mimetic. Then I dissected the molecular basis behind the response. After confirming the effect of TRAIL and Smac mimetic co-treatment *in vitro*, I also evaluate the efficacy of the TRAIL and Smac mimetic co-treatment *in vivo* within the LSL–K-RAS G12D mice.

3.1 TRAIL and Smac mimetics specifically induce apoptosis in KRAS-activated HBE4 and BW1799 cells.

To test if TRAIL and Smac mimetics combination can eliminate the premalignant lung cells, we need to establish an *in vitro* model of the premalignant lung cancer cells. So here we introduced the activating mutant $KRAS^{G12V}$ into the immortalized normal lung epithelia cell lines HBE4 and BW1799. Signaling downstream of KRAS, including Erk and AKT, was increased following constitutive expression of mutant KRAS in HBE4 and BW1799 cell lines (Fig. 9A). Then, to test whether KRAS activation sensitizes

normal lung epithelial cells to TRAIL plus Smac mimetics–induced apoptosis, HBE4 cells transfected with vehicle or mutant KRAS plasmid were treated by Smac mimetic for 30min, then followed by TRAIL treatment or by Smac mimetic or TRAIL treatment individually. After 24 hours post TRAIL treatment, the apoptotic cells were determined with annexin V-FITC apoptosis detection kit. Results showed that HBE4 cells transfected with vehicle plasmid are resistant to apoptosis induced by TRAIL, Smac mimetic or the combination (Fig. 9B), while cells transfected with mutant KRAS are sensitive to TRAIL plus Smac mimetic–induced apoptosis but not the single treatment (Fig. 9B). Similar results were observed in BW1799 cells (Fig. 9C). These results indicate that activation of KRAS specifically sensitizes normal lung epithelial cells to the induction of apoptosis by the combination of TRAIL and Smac mimetic.

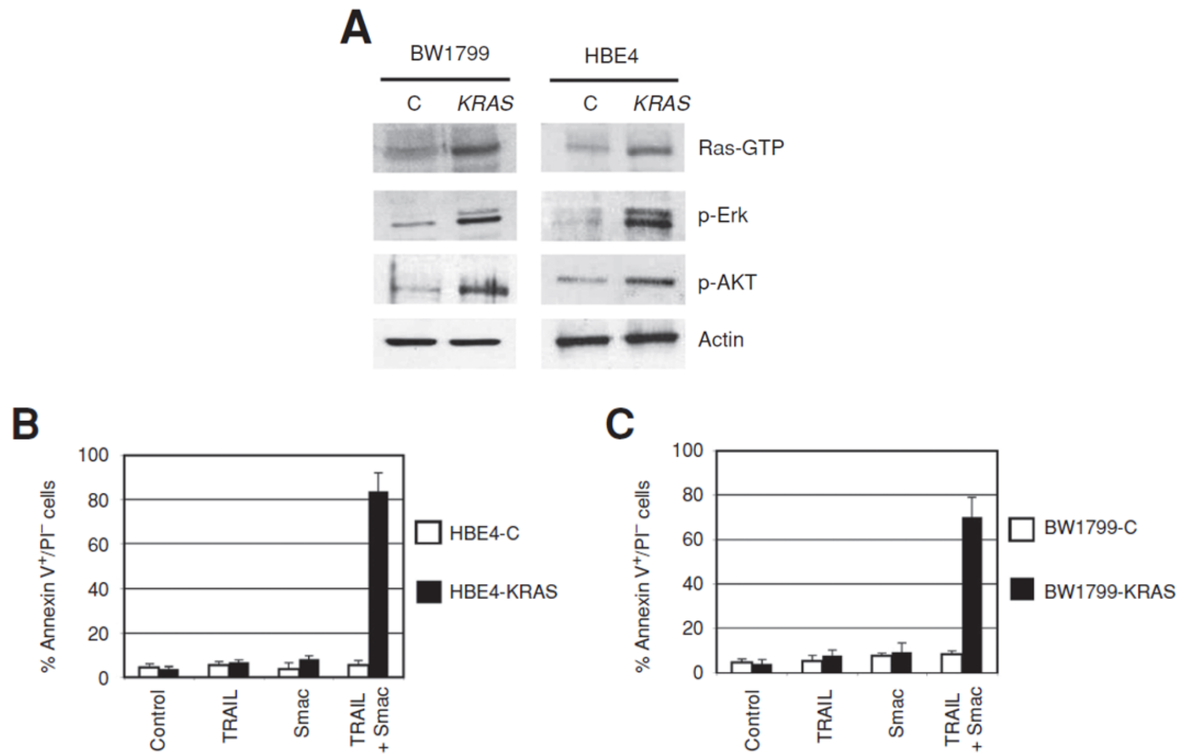

Figure 9. Mutant KRAS activates downstream-signaling pathways and sensitizes normal cells to TRAIL and Smac mimetics. A, overexpression of mutant KRAS and induction of phosphorylation of ERK (p-ERK) and AKT (p-AKT) in BW1799 and HBE4 cells. These cells were infected with either vehicle retrovirus (Vec) or mutant KRAS expressing-retrovirus. B&C, induction of apoptosis by TRAIL and Smac mimetics. HBE4 cells and BW1799 cells expressing mutant KRAS were treated with TRAIL (100 ng/mL) or Smac mimetics (100 nmol/L) or both or control (PBS) for 24 hours. Apoptotic cells (Annexin V^+/PI^-) were counted. The data represent results from 3 independent experiments. Averages and SD are shown.

3.2 Mutant KRAS up-regulates TRAIL receptor levels to facilitate TRAIL-induced apoptosis in normal cells.

Even we hypothesize the synthetic lethality will be generated by combination of TRAIL, RAc and Smac mimetic, previous results showed that the combination of TRAIL and Smac mimetic can induce apoptosis in the normal lung epithelial cells with KRAS activation independent of RAc. Then I tested the role of RAc in normal lung epithelial cells as well as APC deficient colon cells. The results showed that RAc is not essential for TRAIL plus Smac mimetic–induced apoptosis (Fig. 10A). This suggests that KRAS may modulate the expression of TRAIL receptors as RAc does in the APC deficient colon cells. I tested this possibility by examining TRAIL receptor expression by Western blot. Expression of mutant KRAS induced the expression of DR4 and DR5 (Fig. 10B). Mutant KRAS also significantly inhibited the expression DcR2 (Fig. 10B). These results indicate that expression of mutant KRAS enhances the DR4/5 expression while repressing the DcR1/2 expression, which facilitates TRAIL signaling. In other words, mutant KRAS exerts a similar effect on TRAIL receptors to that of RAc and thus eliminates the need for adding RAc to TRAIL plus Smac mimetic for the induction of apoptosis in mutant KRAS cells.

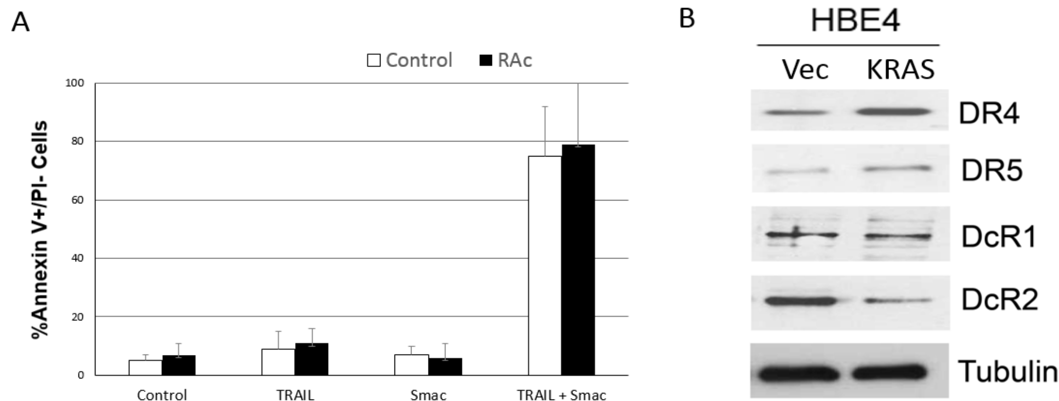

Figure 10. Modulation of TRAIL-receptor expression by KRAS. A, RAc did not enhance TRAIL- and Smac mimetics–mediated apoptosis in HBE4 cells expressing mutant KRAS. B, Effect of KRAS activation on the TRAIL receptors DR4, DR5, DcR1, and DcR2. HBE4 cells were infected with either vector or mutant KRAS-expressing retrovirus.

3.3 Sensitization to TRAIL plus Smac mimetic mainly depends on the activation of the MAPK/ERK pathway.

KRAS functions as an upstream activator of RAF-MEK-ERK and PI3K-Akt signaling pathways, which both can up-regulate the cMyc [25-26]. The activation of KRAS signaling may sensitize normal bronchial epithelial cells to apoptosis induced by TRAIL and Smac mimetic via cMyc activation. To investigate the role of two major KRAS downstream signaling pathways (activation of ERK through RAF and of AKT through PI3K) in contributing to TRAIL plus Smac mimetics–induced apoptosis in mutant KRAS cells, I used MAPK/ERK and AKT inhibitors or dominant-negative mutants to block the signaling pathways in HBE4-KRAS stable cells. Induction of dominant-negative mutants of ERK1/2 inhibited KRAS-mediated ERK activation and TRAIL plus Smac mimetics–induced apoptosis; similarly, U0126, a MEK inhibitor upstream of ERK, also blocked TRAIL plus Smac mimetics–induced apoptosis via inhibition of ERK activation (Fig. 11A and 11B). The inhibition of AKT activation by either

the AKT dominant-negative mutant or Wortmannin, a PI3K inhibitor upstream of AKT, did not attenuate TRAIL plus Smac mimetics–induced apoptosis (Fig. 11A and 11B). These results indicate that the sensitization to TRAIL plus Smac mimetics-induced apoptosis is primarily dependent on activation of the MAPK/ERK pathway.

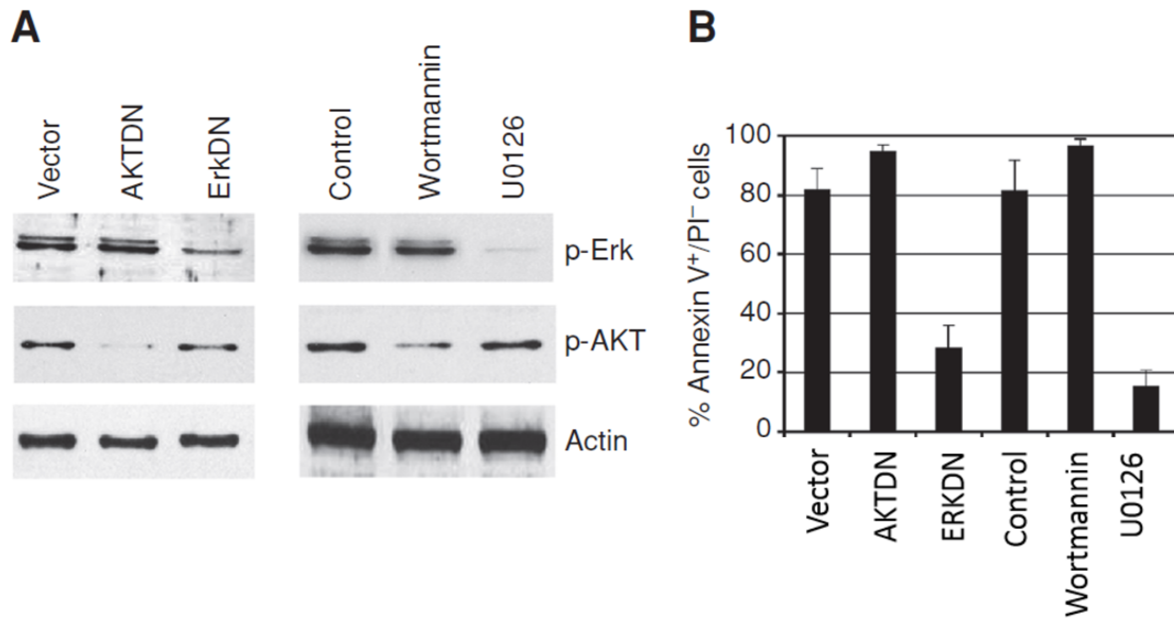

Figure 11. The ERK/MAPK pathway is essential for KRAS-mediated sensitization. A, inhibition of KRAS-mediated activation of ERK and AKT. Phosphorylated ERK (p-ERK) and AKT (p-AKT) were assessed in HBE4-KRAS cells transfected with a dominant-negative AKT mutant (AKTDN) or ERK1/2 dominant-negative mutants (ERKDN) or vector (control), or in these cells treated with the PI3K inhibitor Wortmannin (200 nmol/L) or the dual MEK1 and MEK2 inhibitor U0126 (10 mmol/L) or control (dimethyl sulfoxide, DMSO). B, TRAIL- and Smac mimetics–mediated apoptosis was attenuated by ERKDN and MEK1/2 inhibitors. The dominant-negative mutant transfected or inhibitors treated HBE4-KRAS cells were treated with TRAIL and Smac mimetics for 24 hours. Apoptotic cells (Annexin V^+/PI^-) were counted. The data represent results from 3 independent experiments. Averages and SD are shown.

3.4 Sensitization to TRAIL plus Smac mimetic in oncogenic KRAS–expressing normal epithelial cells is dependent on the regulation of c-Myc and c-FLIP levels.

Even though previous results indicate that the sensitization to TRAIL plus Smac mimetics-induced apoptosis is primarily dependent on activation of the MAPK/ERK pathway, we still do not know if the MAPK/ERK activation regulate the expression of c-Myc, which in turn inhibits the expression c-FLIP to sensitize cells to TRAIL and Smac mimetics. So I first analyzed the expression of c-Myc and c-FLIP in mutant KRAS cells. As we predicted, expression of mutant KRAS resulted in increased levels of c-Myc protein and consequently decreased levels of c-FLIP protein expression (Fig. 12A). I then sought to confirm the significant role of c-Myc and c-FLIP in sensitization to TRAIL plus Smac mimetic. To this end, c-Myc-shRNA was transfected into mutant KRAS cells to knock down c-Myc expression. The transfection abolished KRAS-mediated induction of c-Myc expression and restored c-FLIP expression (Fig. 12B). More importantly, knockdown of c-Myc significantly inhibited TRAIL plus Smac mimetics–induced apoptosis (Fig. 12D). Furthermore, restoring c-FLIP expression by transfecting a c-FLIP–expression plasmid blocked TRAIL plus Smac mimetics–induced cell death in mutant KRAS cells (Fig. 12C and 12D). These data support the ability of KRAS to sensitize normal HBE4 cells to TRAIL and Smac mimetics through activation of c-Myc and the subsequent repression of c-FLIP expression.

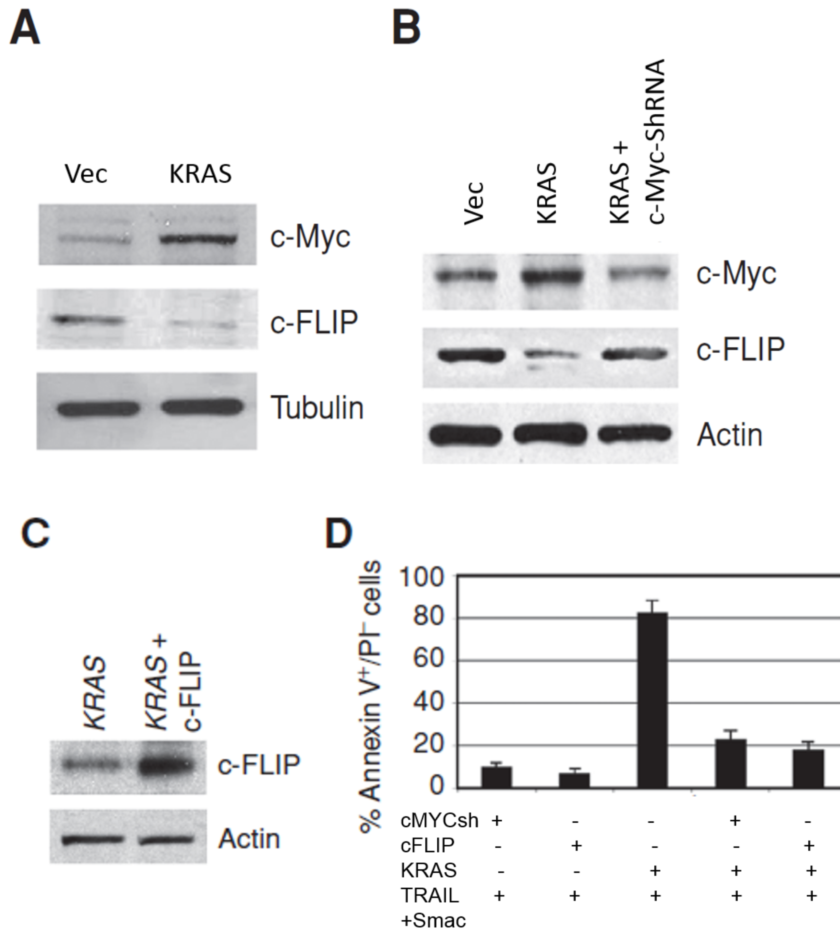

Figure 12. c-MYC and c-FLIP are involved in TRAIL plus Smac mimetics–induced apoptosis in mutant KRAS–expressing cells. A, mutant KRAS activates c-MYC and represses c-FLIP. HBE4 cells were infected with either control or KRAS-expressing retrovirus. B, c-MYC was knockdown in mutant KRAS-expressing cells. HBE4 cells were infected with either control or KRAS-expressing virus or were transfected with c-Myc-shRNA after KRAS infection. C, c-FLIP is overexpressed in in mutant KRAS-expressing cells. HBE4 cells infected with KRAS were transfected with either control vector or full length c-FLIP plasmid. D, apoptosis was attenuated by cMYC knockdown or cFLIP overexpression. HBE4 cells with various infection and transfection combinations were treated with TRAIL and Smac mimetics. Apoptotic cells (Annexin V+/PI-) were counted. The data represent results from 3 independent experiments. Averages and SD are shown.

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3.5 Smac mimetic overcomes the antiapoptotic activity of XIAP to facilitate TRAIL-induced apoptosis.

Previous results had shown that not the single TRAIL or Smac mimetic but only the TRAIL plus Smac mimetic combination can induced the apoptosis in mutant KRAS cells. We hypothesize that Smac mimetic could bind to IAPs, release the caspases and sensitize the mutant KRAS cells to TRAIL induced the apoptosis. But it is also reported that Smac mimetic could activate NF-κB pathway and increase the autocrine TNFα level which may also trigger the apoptosis [148-149]. So I next examined the target of Smac mimetic in sensitizing mutant KRAS cells to TRAIL. I first analyzed XIAP expression in mutant KRAS cells by Western blot. Although expression of XIAP was not significantly affected by mutant KRAS, significantly high levels of XIAP were detected, suggesting that the anti-apoptotic activity of XIAP needed to be inhibited to facilitate TRAIL induced apoptosis (Fig. 13A). To investigate this possibility, I used XIAP-shRNA to inhibit the expression of XIAP in mutant KRAS cells (Fig. 13A). Knockdown of XIAP sensitized mutant KRAS cells to TRAIL-induced apoptosis (Fig. 13B), in the absence of Smac mimetic. These results support the role of Smac mimetic in targeting the anti-apoptotic activity of XIAP to facilitate TRAIL induced apoptosis. This sensitization is independent of intrinsic pathway of apoptosis.

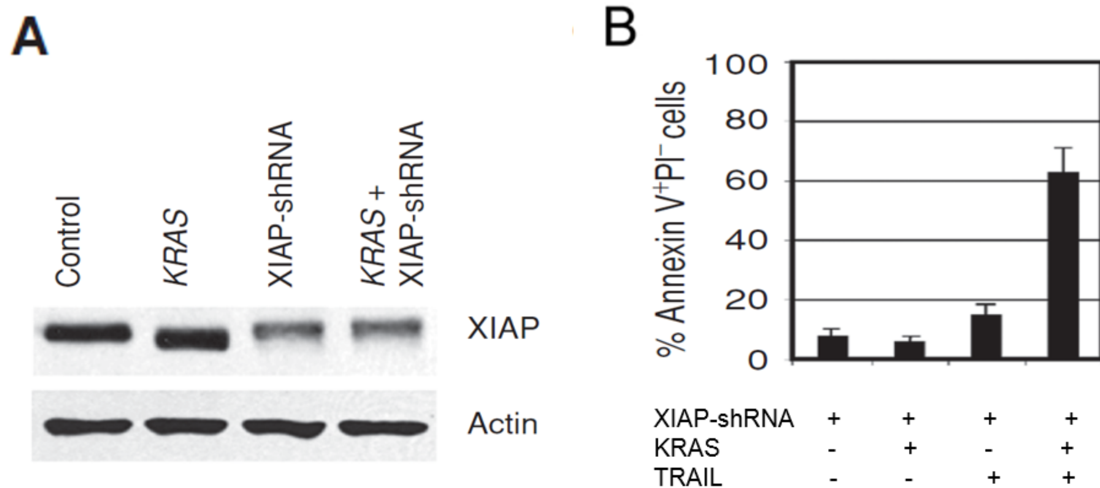


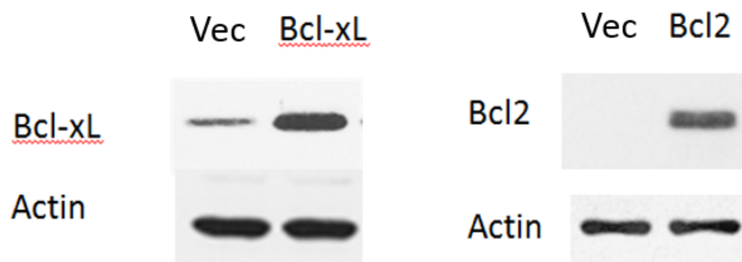
Figure 13. Role of XIAP in TRAIL plus Smac mimetics–induced apoptosis in oncogenic KRAS–expressing cells. A, effect of XIAP knockdown. HBE4 cells were infected with either control or mutant KRAS-expressing virus or were transfected with XIAP-shRNA after KRAS infection. B, induction of apoptosis. HBE4 cells with various infection and transfection combinations were either treated with TRAIL or not treated. Apoptotic cells (Annexin V+/PI-) were counted. The data represent results from 3 independent experiments. Averages and SD are shown.

3.6 Apoptosis induced by the combination of Smac mimetic and TRAIL treatment is independent of intrinsic apoptotic pathway.

It is reported in the literature that caspase 8/10 can cleave BID via TRAIL binding to death receptor and formation of the DISC complex [114]. The cleaved form of BID, tBID, can activate the intrinsic pathway by penetrating the mitochondria membrane and release a group of pro-apoptotic proteins from mitochondria, such as Cytochrome-c and Smac. Pro-apoptotic proteins other than Smac may also contribute to the TRAIL induced apoptosis. To determine whether the intrinsic apoptotic pathway also plays a role in the

apoptosis induced by TRAIL in KRAS mutant cells, Bcl_2 and Bcl-xL constructs were transfected into mutant KRAS HBE4 cells (Fig. 14A). Overexpression of either Bcl_2 or Bcl-xL did not prevent the TRAIL-induced apoptosis (Fig. 14B). These results suggest that the apoptosis activated by TRAIL and Smac mimetic is independent of mitochondrial apoptotic pathway.

A



B

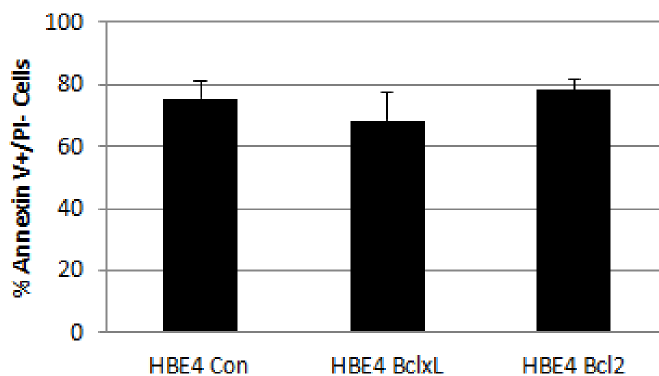
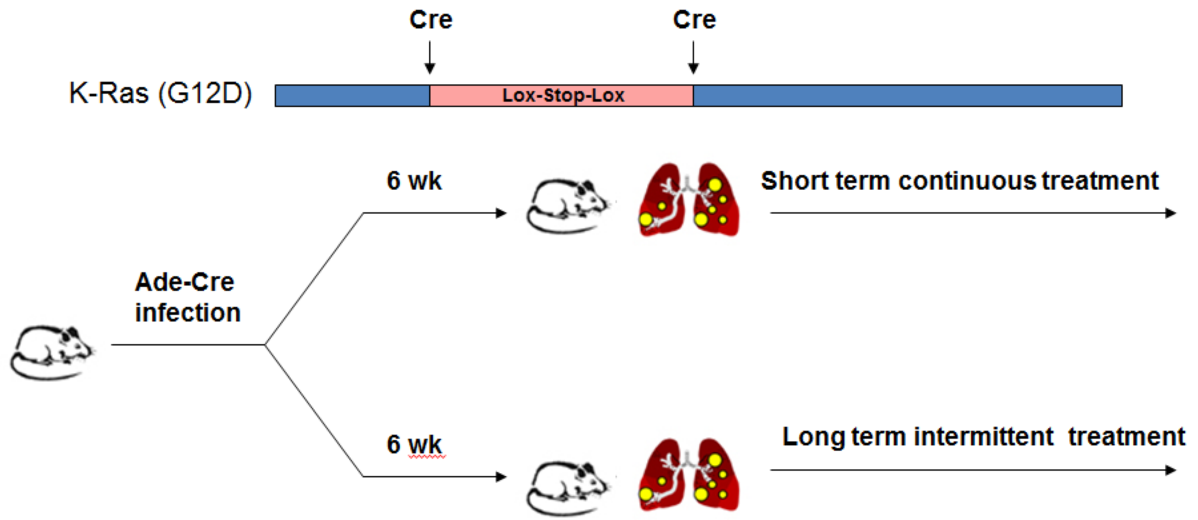


Figure 14. Overexpression of Bcl_2 or Bcl-xL in KRAS mutant HBE4 cells did not rescue the cells from death. A, HBE4 cells were transfected with either vector or Bcl2 or Bcl-xL constructs after KRAS infection. B, Induction of apoptosis. HBE4 cells with various infection and transfection combinations were either treated with TRAIL or not treated. Apoptotic cells (Annexin V+/PI-) were counted. The data represent results from 3 independent experiments. Averages and SD are shown.

3.7 Induction of carcinogenesis in a mouse model of KRAS driven lung cancer.

Previous results have showed that activation of KRAS specifically sensitizes normal lung epithelial cells to the induction of apoptosis by the combination of TRAIL and Smac mimetic *in vivo*. To further test the effect of TRAIL and Smac mimetic-mediated apoptosis for chemoprevention, we first needed to establish a mouse model with KRAS activation, which mimics the process of carcinogenesis in humans. For this purpose, I chose a mouse model of mutant KRAS (KRAS-G12D)–driven lung adenocarcinoma. KRAS-G12D mice carry a conditional allele of oncogenic KRAS-G12D (LSL-KRAS-G12D) and closely mimics the tumorigenesis initiated in humans through somatic KRAS mutation [150]. LSL-KRAS-G12D contains a floxed transcriptional stop element, and infecting the lungs of these mice with AdenoCre virus, a recombinant adenovirus expressing the Cre recombinase, resulted in the expression of mutant KRAS and the development of epithelial hyperplasia of the bronchioles, adenomas, and eventually pulmonary adenocarcinoma [150]. Using the dose of AdenoCre virus mentioned before, I sacrificed the mice at 3 wks and 6 wks post-infection (Fig. 15A). Then their lungs were inflated with formalin and fixed in formalin overnight. Lung sections were analyzed by hematoxylin and eosin (H&E) staining. The histology results show the different stages of carcinogenesis in lung tissue (Fig. 15B). At 3 wks after virus infection, there are only a few hyperplasias while the uninfected lung is free of hyperplasia as the red arrows indicated. At 6 wks after virus infection, the number of hyperplasias continues to increase and there are some areas containing adenoma-like hyperplasia as the yellow arrows indicated.



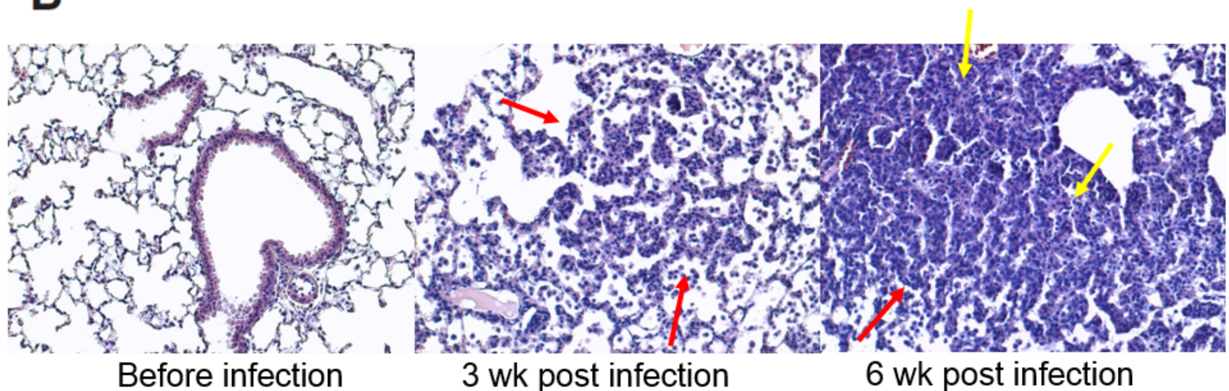


Figure 15. A KRAS lung tumor mice model was established to test the effect of TRAIL and Smac mimetic. A, The mice were infected with AdCre-CaPi coprecipitates (5 - 10^8 pfu) intranasally in two 62.5-mL instillations. Six weeks after infection, the mice were treated either by 3 time continuous treatments or by intermittent treatment, 3 continuous injections a cycle, 3 weeks a cycle, totally 3 cycles. B, the mice were sacrificed before the infection or 3wks or 6 wks after the infection. The lung sections were analyzed by hematoxylin and eosin (H&E) staining.

3.8 Short-term continuous TRAIL and Smac mimetic treatment inhibits lung tumor growth in KRAS transgenic mice.

As TRAIL plus Smac mimetic induces apoptosis in oncogenic KRAS–expressing cells *in vitro*, this combination has the potential for chemoprevention through short term and intermittent therapy. I tested this potential *in vivo* beginning 6wks after the induction of lung carcinogenesis. The mice received 3 consecutive injections of TRAIL plus Smac mimetics within 1 week. Then the mice were sacrificed 1 day following the treatment, and the lungs were examined for evidence of tumors by H&E staining. I used cleaved caspase-3 immunohistochemistry to evaluate induction of apoptosis. TRAIL plus Smac mimetic induced a significant level of apoptosis in lung tumor cells (Fig. 16A). There was no evidence of apoptosis in normal lung sections (Fig. 16A). Treated mice had a significantly decreased number of lung lesions (hyperplasias and adenomas; versus control mice; Fig. 16B). The most dramatic decrease was a 97% reduction in advanced lung lesions (adenomas; Fig. 16B). Therefore, short-term treatment with TRAIL and Smac mimetic inhibited *in vivo* lung tumor growth.

3.9 Intermittent TRAIL and Smac mimetic treatments strongly inhibit lung tumor growth in KRAS transgenic mice.

To test the effect of intermittent TRAIL and Smac mimetics treatment, I treated the mice with 3 cycles of the 3-perweek consecutive treatments within 2 months. The mice were sacrificed 3 days after the last treatments, and the lungs were examined for evidence of tumors by H&E staining. At the time of analysis, most lesions in control mice

were adenomas, whereas most lesions in treated mice were hyperplasias (Fig. 16C). A minor, but statistically significant, reduction was observed in the total number of lung lesions in the treatment group (versus controls; Fig. 16D). More important, the number of advanced lesions (adenomas) was greatly reduced in the treatment group (versus controls; Fig. 16D). The lower number of total lung lesions in Figure 16D compared to that in Figure 16B is likely due to the presence of some adenomas, which were more numerous in Figure 16D, comprising multiple hyperplastic lesions. These results support the ability of TRAIL plus Smac mimetic to induce apoptosis in mutant KRAS cells *in vivo* and support the potential of this combination for therapy-like chemoprevention of human lung cancer.

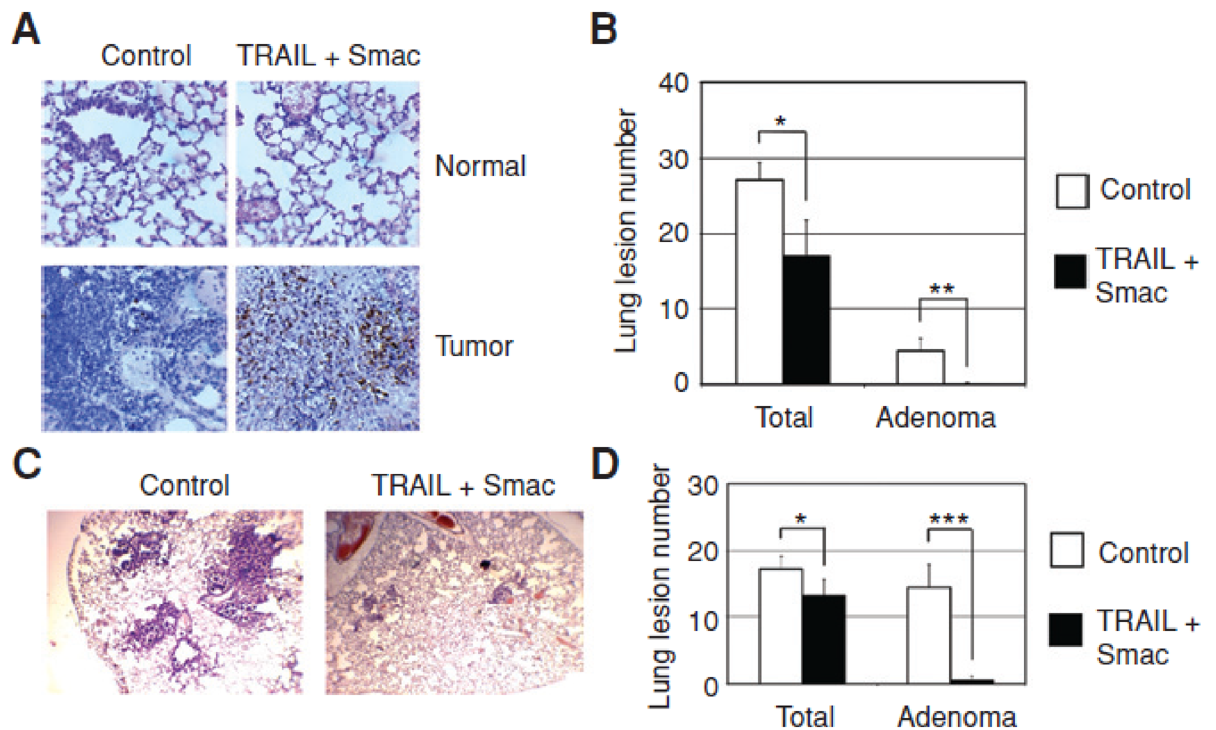


Figure 16. Effect of TRAIL and Smac on KRAS-induced lung tumors in mice. A, TRAIL and Smac mimetic induce apoptosis in lung tumors of LSL-KRASG12D mice. LSL-KRAS-G12D mice were infected with AdenoCre and treated 6 weeks later with PBS (control; n ¼ 6) or received 3 consecutive treatments with TRAIL (3 mg/kg) plus Smac mimetics (3 mg/kg; n ¼ 6) within 1 week. Mice without infection were used as normal controls. Three days after the last treatment, the lung sections were stained with an anti–cleaved caspase 3 antibody. Representative photomicrographs are shown. Caspase 3 staining was detected only in TRAIL/Samc-mimetics treated tumor samples. B, TRAIL and Smac mimetics treatment inhibits lung tumor growth. Lung sections were stained with H&E and lung lesions were counted. The data are derived from counting within serial sections in each mouse. Averages and SD are shown. *, P < 0.05; **, P < 0.001. C, Intermittent treatment with TRAIL plus Smac mimetics inhibits adenoma formation. LSL-KRAS-G12D mice were infected with AdenoCre. Six weeks later, the mice (n = 6 for each treatment group) were subjected to 3 rounds of intermittent treatment in 2 months with either PBS (control) or TRAIL (3 mg/kg) plus Smac mimetics (1.5 mg/kg). Lung sections were stained with H&E; representative photomicrographs are shown. D, Quantification of lung lesions. Serial sections were stained with H&E, and lung lesions were counted. The data are derived from counting within serial sections in each mouse. Averages and SD are shown. ***, P < 0.0001.

In this chapter, we observed that the combination of TRAIL and Smac mimetic specifically induce apoptosis in KRAS activated normal cells. The induction of apoptosis did not require the attendance of RAc because KRAS activation modulates the expression TRAIL receptors as RAc did. The KRAS activation sensitize the normal epithelial cells to TRAIL plus Smac mimetic induced apoptosis by modulating the expression of c-MYC and c-FLIP through the MAPK/ERK pathway. And in the process, Smac mimetic overcomes the anti-apoptotic activity of XIAP to facilitate TRAIL-induced apoptosis.

Then I established a mouse model of mutant KRAS (KRAS-G12D)–driven lung adenocarcinoma to mimic the process of carcinogenesis in humans. At 6 wks after virus infection, the histology analysis showed there were a number of hyperplasias and some adenoma-like hyperplasia. Then short-term TRAIL and Smac mimetic treatments strongly induced apoptosis specifically in the hyperplasia area while the intermittent TRAIL and Smac mimetic treatments dramatically inhibit lung tumor growth in mice.

Combined *in vitro* and *in vivo* results showed that TRAIL and Smac mimetics treatment can specifically and efficiently induced apoptosis in KRAS activated normal lung epithelial cells which could mimic the premalignant cells. The long term intermittent treatment results strongly suggest that this approach could be applied for SITEP based chemoprevention.

CHAPTER 4 Use TRAIL and Smac mimetic for lung cancer treatment

Previous results have shown that for the first time a synthetic lethal interaction among TRAIL, Smac, and constitutive activation of RAS in premalignant bronchial epithelial cells with great efficiency and specificity. As mutational activation of KRAS occurs in 25-30% of NSCLC17, targeting oncogenic RAS activation with the combination of TRAIL and Smac mimetic is a potential new approach for the treatment of NSCLC. So in this chapter I investigated the mechanism of TRAIL plus Smac mimetics induced apoptosis in lung cancer cells for NSCLC treatment. I also tested the efficacy of combination treatment in lung cancer using a tumor xenograft model. Because we also observed some KRAS mutant lung cancer cell lines are resistant to TRAIL and Smac mimetic induced apoptosis. The mechanism of resistance to the combination treatment are explored in some respects.

4.1 TRAIL and Smac mimetic induces apoptosis specifically in KRAS mutant lung cancer cell line

To determine whether the TRAIL and Smac mimetic combination treatment can be used to treat lung cancer, I first tested whether the combination treatment was able to kill lung cancer cell lines. I treated different lung cancer cell lines with and without the KRAS mutation. The panel of lung cancer cell lines with KRAS mutation includes H460, H358 and H157. The panel of lung cancer cell lines with wild type KRAS includes H322 and H661. The results indicate that the panel of lung cancer cell lines with KRAS mutation is more

sensitive to the combination treatment than the panel of lung cancer cell lines with wild type KRAS (Figure 17A&B).

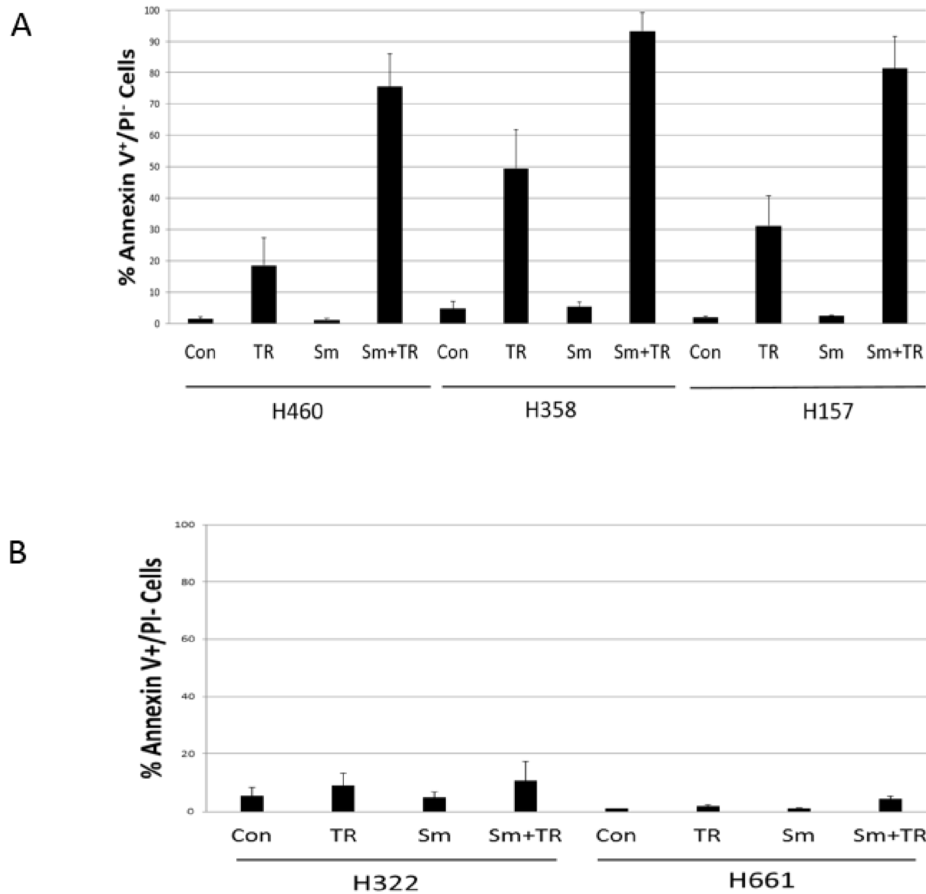


Figure 17. Effect of TRAIL and Smac mimetic on lung cancer cell lines with or without mutant KRAS. A, induction of apoptosis in mutant KRAS cell lines including NCI-H460, NCI-H358 and NCI-H157. B, induction of apopotosis in wild type KRAS cell lines including NCI-H322 and NCI-H661. Cells were treated with TRAIL and Smac mimetics. Apoptotic cells (Annexin V+/PI-) were counted. The data represent results from 3 independent experiments. Averages and SD are shown.

4.2 Expression of mutant KRAS sensitize KRAS wild type lung cancer cell lines to TRAIL and Smac mimetic induced apoptosis.

Previous results showed that the panel of lung cancer cell lines with KRAS mutation is more sensitive to the combination treatment, which suggest that KRAS mutation is relative to the positive response. To further confirm whether KRAS activation sensitizes the KRAS wild type lung cancer cells to TRAIL plus Smac mimetic–induced apoptosis, I introduced the activating mutant $KRAS^{G12V}$ into the KRAS wild type lung cancer cell line NCI-H322 as previously described for the normal cells. Unsurprisingly, constitutive expression of mutant KRAS led to activation of downstream signaling pathways, including phosphorylation of ERK and AKT in NCI-H322 cells (Fig. 18A). As expected, the MAPK/ERK activation regulate the expression of c-Myc, which in turn inhibits the expression c-FLIP (Fig.18A). Because previous data also showed that the KRAS activation enhances the DR4/5 expression while repressing the DcR1/2 expression, which facilitates TRAIL signaling. So we also hypothesize that KRAS activation could modulate the death receptors and decoy receptors level in NCI-H322 lung cancer cells as in normal lung epithelial cells. But the western blotting results is not consist with previous results (Fig. 18A). In NCI-H322 cells, only the DR5 expression level was slightly up-regulated but the DR4 level was not. The decoy death receptors DcR1/2 were not depressed as in in normal lung epithelial cells.

Then, to test whether KRAS activation sensitizes NCI-H322 lung cancer cells to TRAIL plus Smac mimetics induced apoptosis, NCI-H322 cells infected with vector or mutant KRAS retrovirus were treated by Smac mimetic for 30min, then followed by TRAIL treatment. After 24 hours post TRAIL treatment, the apoptotic cells were determined with annexin V-FITC apoptosis detection kit. Results showed that NCI-H322 cells infected with vector virus are resistant to apoptosis induced by TRAIL plus Smac mimetic treatment while expression of mutant KRAS sensitized part of the cells to TRAIL plus Smac mimetic induced apoptosis (Fig. 18B). These results suggest that activation of KRAS may specifically sensitize KRAS wild type lung cancer cell NCI-H322 to TRAIL and Smac mimetic induced apoptosis. Comparing to the results in normal lung epithelial cells, the apoptosis was induced in only

about 40% NCI-H322 lung cancer cells even the c-MYC and c-FLIP levels were regulated as we expected. Considering the death receptors DR4/5 were not up-regulated as in normal lung epithelial cells, it may suggest that death receptors level are one of the key factors to decide the cells response to TRAIL plus Smac mimetics induced co-treatment.

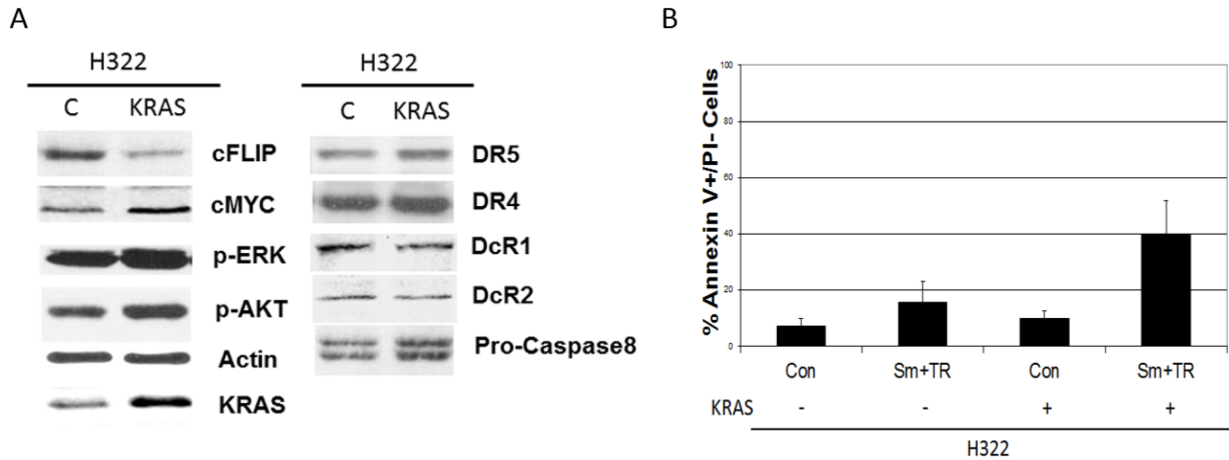


Figure 18. Effect of TRAIL and Smac on wild type KRAS lung cancer cell line after KRAS activation. A, these cells were infected with either vector (vec) or mutant KRAS expressing retrovirus. Activation of KRAS induct phosphorylation of ERK (p-ERK) and AKT (p-AKT), activation of c-Myc and repression of c-FLIP by KRAS in NCI-H322 cells. The results also showed effect of KRAS activation on the TRAIL receptors DR4, DR5, DcR1, DcR2 and pro-caspase 8. B, KRAS-mediated H322 cells were infected with either control or KRAS-expressing retrovirus. H322 cells were infected with either control or KRAS-expressing retrovirus. B, induction of apoptosis by TRAIL and Smac mimetics. H322 cells expressing oncogenic KRAS were treated with TRAIL (100 ng/mL) or Smac mimetics (100 nmol/L) or both or control (PBS) for 24 hours. Apoptotic cells (Annexin V^+/PI^-) were counted. The data represent results from 3 independent experiments. Averages and SD are shown.

4.3 TRAIL and Smac mimetic mediate tumor growth suppression in KRAS activated lung cancer xenograft model via induction of apoptosis.

Previous results showed that lung cancer cell lines with KRAS mutation is more sensitive to the TRAIL plus Smac mimetic treatment. And activation of KRAS also sensitize KRAS wild type lung cancer cell NCI-H322 to TRAIL and Smac mimetic induced apoptosis. Those *in vitro* results showed the efficient anti-tumor effect of TRAIL and Smac combination treatment. To further test the anti-tumor effect of TRAIL and Smac mimetic *in vivo*, I utilized a xenograft tumor model. For this experiment, I injected two different lung cancer cell lines, NCI-H460 and NCI-H322 into nude mice to generate xenograft tumors. NCI-H460 cell line harbors mutant KRAS while NCI-H322 has the wild KRAS. This enables a direct comparison of these two types of lung cancer cell lines with the same treatment.

Since there was no previously published data on the dose of TRAIL and Smac mimetic for *in vivo* lung tumor xenograft models, I used the same Smac mimetic dose (3 mg/kg) but doubled the TRAIL dose (6 mg/kg) compared with the transgenic KRAS mice model. In the NCI-H322 xenograft mice, the growth of tumors was very similar in both control and treated animals. However, in the mice with NCI-H460 xenograft, the treated group exhibited decreased tumor growth compared to the control group (Fig. 19A). At the end of treatment (day 24), the NCI-H322 tumor relative volume increased 8.23 times in control group versus 9.09 times in the mice treated with TRAIL plus Smac mimetics, while the NCI-H460 relateive tumor volume increased 8.48 times in control group compared with only 2.68 times in treated group (Fig. 19B). Despite the small number of animals in this

study, the difference between NCI-H460 control group and treated group is statistically significant, and the trend is clear. These *in vivo* results demonstrate that the combination of TRAIL and Smac mimetic only demonstrates an anti-tumor effect in the NCI-H460 xenograft tumor model, but not in the NCI-H322 xenograft tumor model. Although these data are consistent with the *in vitro* results, I still sought to confirm that the anti-tumor effect is due to apoptosis induced by the TRAIL and Smac mimetic, and not some other mechanism. To determine this, TUNEL staining was performed on the xenograft tumor sections and the results confirm that apoptosis was only induced in the NCI-H460 treated group, not in either the NCI-H322 treated group or the NCI-H460 control group (Fig. 19C). These TUNEL results confirm that the anti-tumor effect was accomplished through apoptosis induced by the TRAIL and Smac mimetic combination treatment.

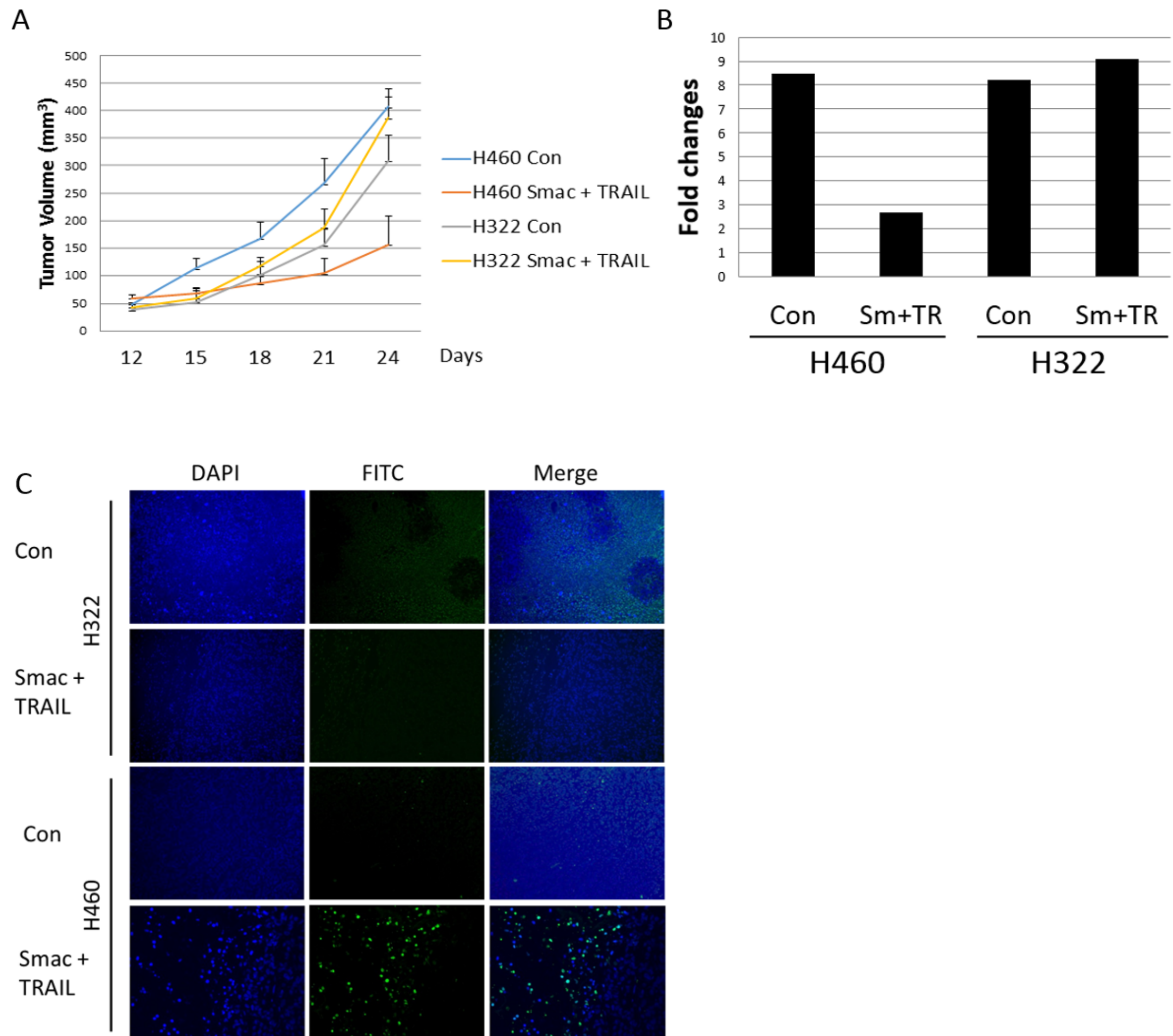


Figure 19. The effects of combination treatment in the *in vivo* xenograft model. A, xenograft tumor growth curves in control and TRAIL plus Smac mimetic treated mice. B, the normalized tumor growth fold at the end of treatment (day 24) in control and TRAIL plus Smac mimetic treated mice. C, the TUNEL staining results of tumor sections in control and TRAIL plus Smac mimetic treated mice.

4.4 Resistance in KRAS mutant lung cancer cell lines may be related to the death receptor expression levels.

Even though TRAIL and Smac mimetic combination treatment exhibited strong killing effects *in vitro* and *in vivo*, it still did not kill all of the cells *in vitro* and it did not cause complete regression of the xenograft tumor. This indicates that there may be additional mechanisms that are inhibiting activation of apoptosis and protecting the cells from death. To investigate possible mechanisms, I first screened additional lung cancer cell lines with KRAS mutations. Among those cell lines, I found that the following cell lines are resistant to TRAIL and Smac mimetic combination treatment to different extents: A549, NCI-H2122 with KRAS mutation and NCI-H2199 with HRAS mutation (Fig.20A).

To investigate the mechanism of resistance to Smac mimetic and TRAIL induced apoptosis in KRAS mutant lung cancer cell lines, I first determined the role of extrinsic and intrinsic apoptosis in response to treatment with TRAIL and Smac mimetic. Overexpression of either Bcl_2 or Bcl-xL did not prevent the TRAIL plus Smac mimetic-induced apoptosis in KRAS mutant lung cancer cell lines NCI-460 and NCI-H358 (Fig. 20B&C). These results are consistent with what I observed in KRAS activated HBE cells. This data combined with reports that Smac alone can induce apoptosis independent of Apaf-1/Cyt C [134], I conclude that TRAIL plus Smac mimetic-induced extrinsic apoptosis is sufficient to trigger downstream caspase activation and cell death, without the involvement of the intrinsic apoptotic pathway. I next evaluated major components of the extrinsic apoptotic pathway in those lung cancer cell lines by western blot (Fig. 20D). The results suggest that

the resistance may not be due to cFLIP and cMyc level as in the HBE cells. In contrast, DR5 and pro-caspase 8 levels are the most likely predictors of responses to TRAIL plus Smac mimetic induced apoptosis.

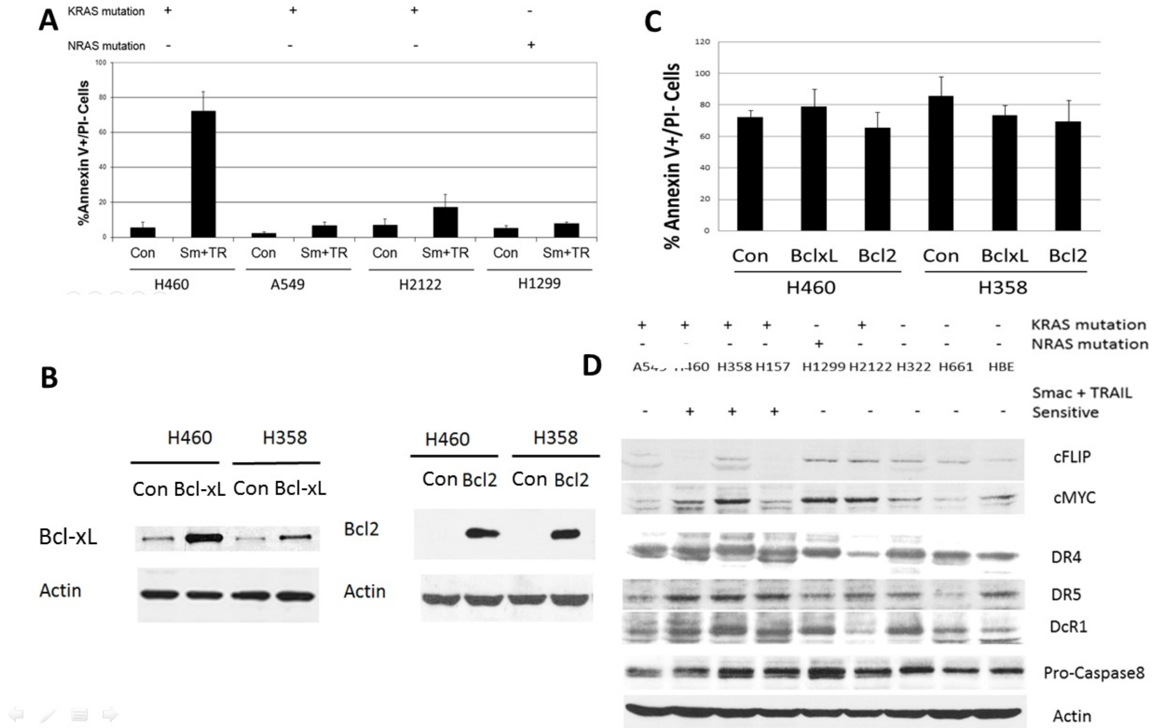


Figure 20. The resistance are highly relative to death receptor DR5. A, Induction of apoptosis. Mutant KRAS cell lines including A549 and NCI-H2122, and mutant NRAS cell lines NCI-1299, were treated with TRAIL and Smac mimetics. Apoptotic cells (Annexin V+/PI-) were counted. B, overexpression of Bcl2 and Bcl-xL in NCI-H460 and NCI-H358 cells. The Bcl2 and Bcl-xL plasmids were transfected into the NCI-H460 and NCI-H358 cells. 24 hour after the transfection, cell lysates were collected. C, induction of apoptosis. NCI-H460 and NCI-H358 with Bcl2 or Bcl-xL overexpression were either treated with TRAIL plus Smac mimetics or not treated. Apoptotic cells (Annexin V+/PI-) were counted. D, Comparison of the extrinsic apoptosis pathway components in different lung cell lines. The levels of death receptors DR4, DR5 and DcR1; Pro-caspase 8, cFLIP and cMyc are investigated by western blotting. All the data represent results from 3 independent experiments. Averages and SD are shown.

4.5 Increase in DR5 expression via 5Fu stimulation or over-expression can overcome resistance to TRAIL and Smac mimetic induced apoptosis.

Previous results showed that death receptors DR5 and pro-caspase 8 levels may determine the cell response to TRAIL and Smac mimetic induced apoptosis. To further confirm the role of DR5 in determining the response to TRAIL plus Smac mimetic, I transfected the DR5 construct into 2 resistant cell lines, A549 and NCI-H2122 (Fig. 21A). The overexpression of DR5 alone induced some apoptosis in NCI-H2122 cells but very little in A549 cells. However, it can sensitize both A549 and NCI-H2122 to TRAIL plus Smac mimetic induced apoptosis (Fig. 21B). This result confirmed that the low DR5 level in resistant cell lines may be a key factor of the resistance to apoptotic induction.

To further study the resistance mechanisms, I also screened the combination of TRAIL with a panel of anti-tumor drugs for the ability to induce apoptosis in lung cancer cell lines. I found that 5-Fluorouracil (5Fu) is the best candidate to synergize with TRAIL to induce apoptosis (Fig. 21C). While studying the mechanism of TRAIL and 5Fu synergy, I found that 5Fu also up-regulates DR5 levels (Fig. 21D), but not caspase-8 or cFLIP levels (data not shown here). The mechanism for 5FU up-regulation of DR5 remained unclear. However, these results still suggest the importance of DR5 in TRAIL induced apoptosis.

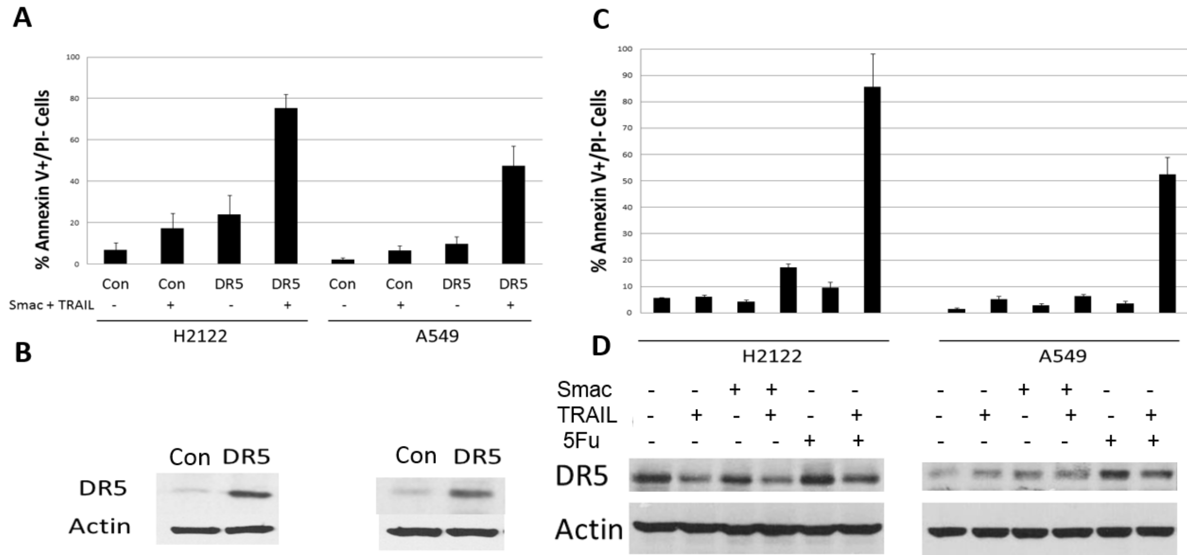


Figure 21. DR5 up-regulation will sensitize the resistant cell lines to TARIL and Smac mimetics induced apoptosis. A, overexpression DR5 in NCI-H2122 and A549 cell lines. DR5 construct was transfected into NCI-H2122 and A549 cells. Lysated were collected 24 hours after the transfecdtion. B, cells tranfected with DR5 or control plasmids were treated with TRAIL (100 ng/mL) or Smac mimetic (100 nmol/L) or both or control (PBS) for 24 hours. Apoptotic cells (Annexin V^+/PI^-) were counted. C, cells treated with 5Fu or DMSO were treated with TRAIL (100 ng/mL) or Smac mimetic (100 nmol/L) or both or control (PBS) for 24 hours. Apoptotic cells (Annexin V^+/PI^-) were counted. D, the DR5 level in cells treated with 5Fu increased. The cells were first treated with 5Fu for 24h, then were treated with TRAIL (100 ng/mL) or Smac mimetic (100 nmol/L) or both or control (PBS) for another 24 hours. Cell lysates were collected. All the data represent results from 3 independent experiments. Averages and SD are shown.

In this chapter, to explore the anti-cancer effect of the combination treatment, I first tested the responses of different lung cancer cell lines to the TRAIL and Smac mimetic treatment. Results showed that several KRAS mutant lung cancer cell lines are sensitive to TRAIL and Smac mimetic induced apoptosis while the KRAS wild type lung cancer cell lines are not. KRAS activation in the KRAS wild type lung cancer cells NCI-H322 can sensitize part of cells to TRAIL plus Smac mimetic induced apoptosis. *In vivo* experiment results showed

that TRAIL and Smac mimetic treatments strongly inhibit mutant KRAS xenograft tumor growth in mice. All the results suggested that the TRAIL and Smac mimetic combination treatment has great anti-cancer effect.

But, the TRAIL and Smac mimetic combination treatment can not completely inhibit the tumor growth in mice. And I also observed that some KRAS or NRAS mutant lung cancer cell lines are resistant to TRAIL and Smac mimetic induced apoptosis. To enhance the anti-cancer effect of the approach, I explored the possible mechanism of resistance. The results suggested that the resistance might be relative to the death receptors level in lung cancer cells. Further study showed that the resistance could be overcome by 5-Fluorouracil or overexpression of DR5. That confirmed the key role of DR5 in determining the lung cancer cells response to TRAIL induced apoptosis.

CHAPTER 5 Summaries and Discussion

5.1 Specific targeting of KRAS mutation based on synthetic lethality.

For the first time, my results demonstrate that a synthetic lethal interaction exists between TRAIL, Smac mimetic, and constitutively active KRAS in premalignant bronchial epithelial cells and malignant lung cancer cells (Fig. 22). This synergy relied on the following mechanisms: KRAS up-regulation of c-MYC and thus repressed c-FLIP expression; KRAS up-regulated death receptors and down-regulated decoy receptors; and Smac mimetic repression of the apoptotic inhibitory effect of IAPs (particularly XIAP). This synthetic lethal interaction made it possible to eliminate KRAS-activated premalignant lung cells or mutant KRAS lung cancer cells using TRAIL plus Smac mimetic with great efficacy and specificity. Normal cells are not sensitive to the combined treatment because of the high level of cFLIP and decoy receptors. This is the rationale for pursuing the treatment or chemoprevention of KRAS mutant lung cancer. As mutational activation of KRAS occurs in approximately 25-30% of NSCLC and there is currently no good method for treating NSCLC with KRAS mutation, targeting oncogenic KRAS activation with the combination of TRAIL and Smac mimeti is potentially a new approach for the therapy and chemoprevention of NSCLC; this potential is strongly supported by my *in vivo* animal studies.

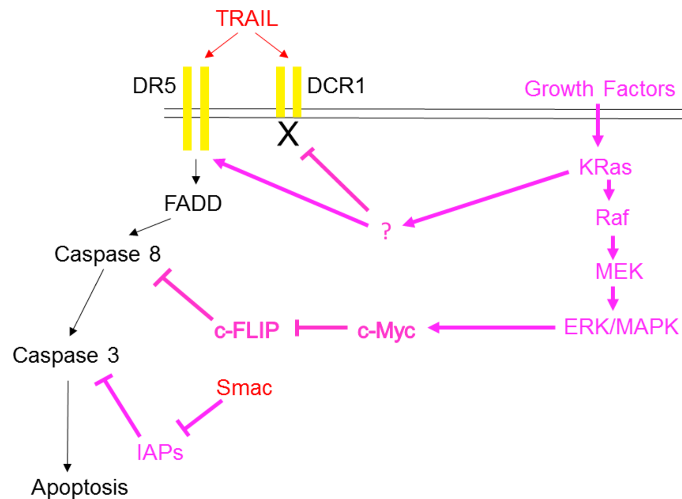


Figure 22. Synthetic lethality targets the KRAS mutant cells. This figure shows that KRAS activation up-regulates cMYC and thus represses cFLIP; Smac mimetic inhibits IAPs; KRAS also modulate the Death receptors and decoyed receptors in unknown mechanism. The synergy can sensitize the cells to TRAIL induced apoptosis.

5.2 SITEP-based lung cancer chemoprevention via synthetic lethality against KRAS mutation.

The concept of synthetic lethality is to generate the cell death only when there are both mutations in synthetically lethal gene pairs, while a single mutation of either gene is compatible with viability. People have begun using this concept to develop cancer-specific cytotoxic drugs in recent years. There are three advantages of synthetic lethality for cancer chemoprevention and therapy. First, synthetic lethality focuses on inducing apoptosis instead of inhibiting some oncogenic pathways, which directly kill the cells in a short time without leaving creating survival pressure for the cancer cells. This decreases the possibility of selection of more resistant malignant cancer cells. Second, cancer-specific mutation makes the associated synthetic lethality interactions very specific and selective.

The interactions will not harm the normal cells that lack the cancer-specific mutation while killing of cancer cells with the mutation, which means this approach should improve toxicity and decrease side effects in patients. Third, while using the synthetic lethality as a prevention strategy, it is not necessary to constitutively administer agents. Applying treatment periodically would eliminate the premalignant cells and decrease the cancer risk, which further decrease the toxicity and the possibility to generate the resistance. These advantages lead to the idea of short-term intermittent therapy to eliminate premalignancy (SITEP), which periodically reduces premalignant tumor cell numbers with short interventions to substantially inhibit or delay cancer development.

Compared to the current chemoprevention strategies, SITEP should reduce the potential side effects and cost associated with long-term drug administration. It also could be predicted that the intermittent treatment would decrease the possibility of generating drug resistance by selectively killing premalignant cells without inducing metabolic changes in adjacent cells.

5.3 Obstacles to apply this new strategy into clinical trial for KRAS mutant NSCLC

Even though the *in vitro* and *in vivo* results showed a promising trend, there are still a number of obstacles in translating this strategy into clinical trials. Both rhTRAIL and various Smac mimetics have been tested separately in a series of clinical trials and showed very little toxicity in patients [125, 145]. But that does not guarantee that the

combination of TRAIL and Smac mimetic are not toxic to patients. Further experiments are required to evaluate the potential toxicity of this combination.

For the purpose of chemoprevention, not only are powerful killing effects required, but also low toxicity and few side effects. This means that lower dosage and longer intervals between the two treatment cycles would be best. To keep the balance of sufficient killing effect and low toxicity, further research needs to be carried out to test different dosage combination of TRAIL and Smac mimetic, including additional intermittent periods and modified TRAIL or agonistic antibodies.

In this project, it is found that KRAS activation can modulate the death receptors DR4/5 and decoy receptors DcR1/2 level and facilitate the TRAIL triggered apoptosis. So the attendance of RAc for generating the synthetic lethality with TRAIL and Smac mimetic is not required here. It is also reported by other literature that DR4/5 levels are always high in KRAS mutant tumors [151]. But the mechanism of KRAS activation regulate DR4/5 and DcR1/2 remains unclear (Fig. 22). There is study that reported the cMYC can up-regulate DR5 level [152]. There is also another study that reported the up-regulation of DR5 by KRAS activation depended on P53 [153]. But the study in my lab showed that cMYC knockdown did not abolish the modulation of DR5 by KRAS. Also the DR5 levels in different lung cancer cell lines are not consistent with the condition of KRAS activation or P53 status (Fig 20). Those results suggested there is unknown mechanism behinds it. Understanding the regulation mechanism will facilitate application of TRAIL based treatment or chemoprevention.

Previous results showed that up-regulation of DR5 by direct overexpression or indirect stimulus could sensitize the resistant NSCLC cells to TRAIL induced apoptosis. Those results suggested that DR5 level or DR5/decoy receptor ratio may be the key factor to determine the cell response upon TRAIL treatment. If we can get the profiles of death receptors and decoy receptors in the patients, we may predict the patient's response to the TRAIL and Smac mimetic combined treatment. Positively modulating the DR5 level also could be set as the criteria when we screen the possible candidates which may have the synergetic effects with TRAIL, just like the RAc or 5Fu.

In this project, I introduce the $KRAS^{G12V}$ mutant plasmids into normal lung epithelial cells or wild type KRAS lung cancer cell lines. But the $KRAS^{G12V}$ mutation could not represent all the KRAS mutations because it is reported that there are codon 12, 13 mutations in NSCLC and different mutations on the same codon, such as G12V and G12D mutations [29]. It is reported that different mutations may activate different downstream signals which means the mutations other than G12V may cause different response of cells to the TRAIL plus Smac mimetic combination treatment[162]. So I need to introduce different KRAS mutations into cells and test the response. This issue also should be concerned for the animal model, which is KRAS-G12D mice.

Some KRAS mutant lung cancer cell lines showed resistance to synthetic lethality, while others did not. Resistance may come from the diverse genetic background of different cancer cells, which is caused by genome instability. I have demonstrated that the level of death receptors is critical to the response to Smac mimetic. The DR4/5 levels

are very low in some cell lines. Also the deficiency of downstream key effectors, such as caspase 8, will protect the cells from TRAIL induced apoptosis. Although NCI-H460 cells are very sensitive to TRAIL plus Smac mimetic treatment, xenograft tumors of this cell line cannot be completely inhibited by this combination. One possible explanation is the limited activity due to very short half-life of the rhTRAIL, only 5-10 minutes when injected i.v. [154]. To overcome this, some groups have tried to use large proteins coupled to TRAIL to increase its stability. Some progress has been made, but increasing toxicity with this approach remains a problem [155]. Agonistic antibodies to TRAIL-R2 (anti-DR5) have been tested, but the outcomes are not encouraging [125]. This may be due to the poor accessibility of large antibodies to tumor tissue.

Whether for therapy or chemoprevention, we need to first target a specific population who are most likely to response to treatment. According to the principle of synthetic lethality, KRAS mutation is one of the key factors that predict response to Smac mimetic and TRAIL, but KRAS mutations occur in both tobacco users and non-smoking patients, which makes selection of a target population more difficult. Among the tobacco users there are current or prior smokers. Is the KRAS mutation the only criteria for those different populations? Are other mutations necessary or exclusive for sensitizing cells to the treatment? Are there any biomarkers or risk factors that can be used to assess the potential effects? These questions remained unanswered so far and they need to be addressed in the further development of SITEP.

5.4 Strategies to overcome resistance.

With continual treatment, it is difficult to avoid the development of resistance due to the selection of malignant and/or drug-resistant cells. The inherent genomic instability of cancer allows cells to develop strategies to inhibit cell death, such as silencing/attenuating death signaling proteins, or over-expression of anti-apoptotic proteins.

To overcome these blockades, we have to activate death signaling pathways while inhibiting anti-apoptotic proteins. In the case of TRAIL plus Smac mimetic, one strategy is to search for agents that modulate the extrinsic signaling molecules. As I mentioned before, positively modulating the DR5 level could be a standard to screen the possible candidates, among which is the 5Fu that could up-regulate DR5 expression. The work in my lab also suggested that 5Fu could increase DR5 distribution on the cell membrane and some specific region, lipid rafts. The combination of TRAIL and 5Fu showed more powerful killing effect than the combination of TRAIL and Smac mimetic *in vitro* and *in vivo*, especially in those resistant cell lines (data not shown here)

Even though my studies demonstrated that TRAIL plus Smac induced apoptosis independently of the intrinsic apoptotic pathway, it is likely that activation of the intrinsic apoptotic pathway will enhance extrinsic apoptosis. So the addition of agents that can penetrate the mitochondria may be another good strategy to overcome resistance. There are several Bcl-2/Bcl-xL inhibitors being studied in clinical trials that may serve as partners to enhance TRAIL plus Smac mimetic induced apoptosis [163-165].

5.5 Prospective studies

For the coming next step, I will focus on solving the questions and obstacles mentioned in the previous discussion part. First we need to completely understand how KRAS modulate the death receptors and decoy death receptors levels, which might be the key factor to determine the cell response to TRAIL triggered apoptosis. The work in my lab had shown that the modulation of death receptors by KRAS is mainly based on the transcription level. So my lab had cloned different promoter and regulation region of death receptors into reporter vector. Then we can test which downstream signals of KRAS mainly accounts for regulating the transcription of death receptors. After understanding how KRAS modulate the death receptors, we could enhance the modulation and even sensitize the resistant cell to TRAIL induced apoptosis.

Previous results showed that the premalignant cells or lesions all response well to the TRAIL plus Smac mimetic treatment while there are more resistances in cancer cells due to the genome instability. So it suggests that the current approach has more great potential in cancer prevention other than cancer treatment. But, we should pay more attention on the concerns of drug selection and drug resistance before applying the current approach into clinical trials. In this project, I applied the SITEP treatment on the infected mice for about 3 months and it showed the efficient effect. But the treatment period may be till not long enough to evaluate the possible accumulating drug resistance. So I plan to keep the SITEP treatment for longer time and get the Kaplan-Meier survival curves, which may reveal the possible resistance.

Besides the above unsolved issues, one of the most difficult obstacles in cancer treatment might be the genomic instability, which generate the diverse genetic background of cancer cells and the resistance to the chemotherapy. So we need a mice model to investigate if there will be resistance upon to the TRAIL and Smac mimetic combination treatment after losing the genomic stability. Now in my lab, we have crossed the conditional P53 knockout mice with LSL-Kras^{G12D} mice. After the mice were infected with AdenoCre virus, the wild type P53 is knocked out and the mutant KRAS is activated. The histology analysis showed that the adenoma formed faster after the adenovirus infection and suggested the tumorigenesis process is accelerated in the mice. So we plan to use this new model to mimic the genomic instability with KRAS activation to test our new approach. Previous results have shown that endogenous DR5, cMYC and cFLIP levels varied in different lung cancer cell lines with KRAS mutation. That also may be due to the genomic instability. To further investigate how genomic instability will affect the cell response to the treatment and determine which factors play the key role in sensitizing the cells to TRAIL induced apoptosis, we will test more lung cancer cell lines and use the database to analysis the relevance of TRAIL sensitivity and genetic information, such as the KRAS mutation profiles.

The long term and final goal of these studies is to develop a new approach for targeting KRAS mutant NSCLC for therapy and chemoprevention. The specificity of this treatment relies heavily on the activation KRAS. In patients who have a tumor biopsy, it is not difficult to check the genetic backgrounds of the tumor. Indeed, for patients who have undergone surgery and are found to have activated KRAS, this strategy may be an effective

way to eliminate the remaining cancer cells or metastatic cells, and prevent tumor recurrence. However, in the case of chemoprevention, the question is how to select patients, or how to identify the high-risk populations? One identifiable group of high risk people are heavy smokers. It has been reported in numerous studies that KRAS mutation is always associated with heavy smoking and the frequency of KRAS mutation is high in this population. So this group might be a good candidate for KRAS targeted chemoprevention. Further evaluation should be taken on the heavy smokers for the combined treatment.

For the non-smokers or prior smokers, even though the risk of developing cancer is low, there is still the possibility of spontaneous KRAS mutation. Since the KRAS mutation is an early event in carcinogenesis and the activation of KRAS will change the downstream signaling, it is expected that there would be some small change of proteins in the circulation. These proteins could be used as predictive makers for the success of synthetic lethality based chemoprevention. The development of microarray techniques and proteomics makes it possible to identify good targets from blood samples as biomarkers to predict KRAS mutation or the response to treatment.

The synthetic lethality of TRAIL and Smac mimetic is based on the mutation but not on the organ site. So this approach could be applied in other cancer types with common KRAS mutations, such as pancreatic cancer, of which 90% are reported to harbor KRAS mutations. Besides cancers with KRAS mutation, cancers that are associated with the activation of growth factor signaling, such as EGFR, which leads to KRAS activation, or cancers with activated downstream effectors of KRAS, such as BRAF, could also be

potential targets for this approach. In support of this some preliminary data from others in our lab has shown that EGFR mutant lung cancer cell lines are sensitive to the combination treatment.

In recently years, personalized cancer therapy and cancer prevention have been a very hot area of research. Synthetic lethality based therapy or prevention could be an important part of the personalized treatment, which provides a strong effect, low toxicity and low costs in selected high-risk patients or populations.

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VITA

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