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Aamir Ahmad
Shirish Gadgeel *Editors*

Lung Cancer and Personalized Medicine

Current Knowledge and Therapies

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Preface

Lung cancer is one of the most common causes of cancer-related deaths in the world, affecting millions of individuals. This volume, *Lung Cancer and Personalized Medicine: Current Updates and Therapies*, comprehensively reviews lung cancer as a disease, details the current state of our knowledge, and showcases the promising novel strategies being pursued. With a greater understanding of lung cancer, we can better appreciate the heterogeneity among lung cancer patients. It is now more evident than ever before that a "one-size-fits-all" approach is not an effective means of clinical management of patients. It is critical to understand every lung cancer patient as an individual, the unique genomic make up an individual patient possesses, and the unique opportunities that such understandings present in developing a treatment plan to which an individual is most likely to respond—that is, "personalized management," the focus of this volume. Chapter 1 provides a commentary on lung cancer statistics. It is important that we remind ourselves of the threats posed by lung cancer. It is the leading cause of cancer-related mortality in men in the USA and throughout the world. In women, it is the leading cause of cancer-related deaths in the USA, but ranks second worldwide. This chapter provides a detailed overview of variations in lung cancer rates and trends in the USA as well as globally. Chapter 2, which addresses the epidemiology of lung cancer, touches upon the various risk factors that may predispose an individual to the disease. Availability of putative risk prediction models will, undoubtedly, change the personalized care of lung cancer patients. Cigarette smoking has always been considered a major factor contributing to lung cancer. However, a substantial number of lung cancer patients never smoked. As a separate entity, lung cancer among nonsmokers will rank as the seventh leading cause of cancer-related deaths, which is discussed in Chapter 3.

Chapter 4, on immune therapy, addresses the recent advances in immune responses in lung cancer patients—previously believed to be irrelevant to lung cancer treatment. This chapter discusses the various immunotherapeutic approaches for treatment of lung cancer patients, including those in clinical trials. With the realization that angiogenesis is frequently upregulated in lung cancer patients, the focus on antiangiogenic agents is of relevance to personalized therapy of lung cancer patients—which is discussed in Chapter 5, which outlines the progress as well as

challenges associated with the antiangiogenic therapy in non-small cell vs. small cell lung cancers. Chapter 6 discusses the targets of personalized therapy in lung cancer, namely, the tyrosine kinases, with a focus on epidermal growth factor receptor (EGFR) mutational status. With such important role of tyrosine kinases in lung cancer progression, tyrosine kinase inhibitors have been studied in considerable detail, but acquired resistance to these inhibitors remains a major clinical challenge. These challenges and the mechanisms of resistance to EGFR-targeted therapies are discussed in Chapter 7, which also touches upon the novel strategies to overcome the resistance. Chapter 8 focuses on KRAS-mutant lung cancers that make up approximately a quarter of all lung cancers. KRAS mutations have also been linked to EGFR-resistance, and their importance in lung cancer progression is increasingly being realized. Chapter 9 focuses on anaplastic lymphoma kinase (ALK), another tyrosine kinase whose chromosomal rearrangement results in aggressive lung cancers. The evolving knowledge on ALK-rearranged lung cancers is of relevance to the personalized management of patients. Rounding up our knowledge on the resistance to current therapies, Chapter 10 summarizes the various chemotherapy options available for lung cancer patients and the associated resistance pathways. Finally, Chapter 11 summarizes the chemistry of metal-based drugs, particularly those based on platinum, ruthenium, gadolinium, and iron compounds. A better understanding of metal-based compounds promises to deliver novel targeted drugs that can potentially be personalized for individual patients.

Part II of this volume, available as a separate volume, addresses the many novel and emerging therapies that can potentially change the way individual lung cancer patients are treated in clinics. Combined, the two volumes provide a detailed overview of topics that are critical to the personalized management of lung cancer.

Detroit, MI, USA

Aamir Ahmad
Shirish Gadgeel

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We, the editors, thank all the authors who worked hard and contributed their knowledge to this volume. Our special thanks to Springer for entrusting us with this exciting project. Our contacts at Springer Publishers—Fiona Sarne at the start of project and Joy Bramble at the end of the project—made sure that we stayed on course; their enormous help is highly appreciated.

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Shirish Gadgeel is a professor in the Department of Oncology at Karmanos Cancer Institute/Wayne State University in Detroit. He is the co-leader of the Molecular Therapeutics Program of Karmanos Cancer Institute and the leader of the Multidisciplinary Thoracic Oncology team. Dr. Gadgeel is a member of several professional societies, including the American Society of Clinical Oncology and the International Association for the Study of Lung Cancer. He currently serves on the Editorial Board of the journals *Cancer* and *Clinical Lung Cancer*. Dr. Gadgeel is a principal investigator of several trials focused on the treatment of lung cancer and has published more than 80 scientific papers.

Lung Cancer Statistics

Lindsey A. Torre, Rebecca L. Siegel, and Ahmedin Jemal

Abstract Lung cancer is the leading cause of cancer death among both men and women in the United States. It is also the leading cause of cancer death among men and the second leading cause of cancer death among women worldwide. Lung cancer rates and trends vary substantially by sex, age, race/ethnicity, socioeconomic status, and geography because of differences in historical smoking patterns. Lung cancer mortality rates in the United States are highest among males, blacks, people of lower socioeconomic status, and in the mid-South (e.g., Kentucky, Mississippi, Arkansas, and Tennessee). Globally, rates are highest in countries where smoking uptake began earliest, such as those in North America and Europe. Although rates are now decreasing in most of these countries (e.g., United States, United Kingdom, Australia), especially in men, they are increasing in countries where smoking uptake occurred later. Low- and middle-income countries now account for more than 50 % of lung cancer deaths each year. This chapter reviews lung cancer incidence and mortality patterns in the United States and globally.

Keywords Lung cancer • Cancer incidence • Age-standardized rate (ASR) • Cancer mortality • Five-year relative survival • Cancer statistics • Age • Race/ethnicity • Socioeconomic status (SES) geographic variation • Trends • United States • Global • International • Global patterns • Cancer burden

Introduction

Lung cancer was rare before the twentieth century, [1] but is now the leading cause of cancer death in both men and women in the United States, accounting for 27 % of cancer deaths in 2014 [2]. Lung cancer is also the leading cause of cancer death in men and the second leading cause of cancer death (after breast cancer) in women worldwide [3]. It was estimated that 1.8 million new lung cancer cases and 1.6 million

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lung cancer deaths occurred in 2012 worldwide, accounting for about 19 % of all cancer deaths [3]. Worldwide variation in the lung cancer burden and trends are primarily driven by historical differences in the uptake and reduction in tobacco use [4].

Common Indicators in Cancer Statistics

Incidence

Cancer incidence is the number of newly diagnosed cancer cases in a population during a specific time period, usually expressed as a rate per 100,000 persons. The numerator includes only cases diagnosed during the given time period, and only primary sites (i.e., metastatic cancers are not counted). The denominator includes only the population at risk for that type of cancer. For example, males would not be included in the denominator for cervical cancer incidence rates, because they are not at risk for cervical cancer.

Age-standardized rates (ASR) are used to compare cancer occurrence between two or more populations with different age structures. It is necessary to account for differences in population age distributions because the frequency of cancer generally increases with age (except for some types of cancers in children). For instance, crude (unstandardized) lung cancer incidence rates are much lower for men in Alaska (a young population) compared to men in Florida (an older population); however, once they are age adjusted, the rates are virtually the same. Age-standardized rates are constructed by taking a weighted average of the rates in each 5 year age group, where the weights are the proportion of persons in that age group in a defined "standard population."

Mortality

Cancer mortality is the number of cancer deaths in a population during a given time period, usually expressed as a rate per 100,000 persons. The numerator includes only deaths which occurred during the given time period, and the denominator includes only the population at risk for that type of cancer. Cancer mortality rates reflect both incidence and survival. For cancers with universally high case fatality, such as lung and pancreatic cancers, mortality rates may sometimes be used as a proxy for incidence rates.

Survival

Cancer survival is the length of time a person lives following cancer diagnosis. Relative survival represents the percentage of cancer patients who are living after a specified time period since cancer diagnosis compared to the expected survival of a cancer-free population of the same age, race, and sex.

Data Sources

Incidence and Mortality in the United States

Incidence rates for 2006–2010 were obtained from the North American Association of Central Cancer Registries (NAACCR)'s Incidence-CiNa Analytic File [5]. The file contains incidence data from the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute and the National Program of Cancer Registries (NPCR) of the Centers for Disease Control and Prevention (CDC). Together, SEER and the NPCR collect cancer incidence data for the entire United States population [6]. Incidence trends for whites and blacks were based on data from SEER (9 registries), covering 1975–2010; for other racial/ethnic groups, SEER (13 registries) data covering 1992–2010 were used. Five-year survival rates were based on cases diagnosed from 2003 to 2009 and followed through 2010 in SEER areas (18 registries).

Mortality data for the United States are obtained from the CDC's National Center for Health Statistics (NCHS) through the SEER Program's SEER*Stat database [7]. The accuracy of recording lung cancer as an underlying cause of death is high in the United States, with death certificates capturing about 89 % of lung cancer deaths in one study [8]. All incidence and mortality rates are age-standardized to the 2000 United States standard population.

Global Incidence and Mortality

Incidence and mortality rates for 2012 were obtained from GLOBOCAN 2012, published by the International Agency for Research on Cancer (IARC). GLOBOCAN estimates cancer incidence and mortality rates in each country of the world using different methods depending on the accuracy and availability of data [9]. Coverage of population-based cancer registries ranges from 1 % in Africa, 6 % in Asia, and 8 % in Latin America to 42 % in Europe, 78 % in Oceania, and 95 % in North America [10]. Mortality data are available for about one third of the world population, and are generally of higher quality in high-income countries [11]. IARC also makes available historic incidence and mortality data in its Cancer Incidence in Five Continents database [10] and World Health Organization Cancer Mortality Database [12]. Global incidence and mortality rates were age-standardized to the 1960 world standard population, and therefore cannot be compared to the United States rates which are generally age-standardized to the 2000 United States standard population.

Lung Cancer Patterns in the United States

Lung cancer rates and trends in the United States vary dramatically by demographic and geographic characteristics such as sex, age, race/ethnicity, state, and socioeconomic status, with incidence and mortality showing generally similar patterns because of

the low survival rate [13]. Most of these differences reflect differences in smoking patterns, [4] with lung cancer death rates beginning to increase at the population level two to three decades after widespread smoking has begun and peaking three to four decades after peak smoking in the population [14]. Cigarette smoking is by far the most important risk factor for lung cancer; 82 % of lung cancer deaths in the US are due to smoking [15].

Age

Lung cancer takes decades to develop after smoking initiation, and is thus rare before age 30 and peaks in the elderly (Fig. 1). Lung cancer rates tend to drop off after around 80 years, likely due to competing mortality from other causes or diminished accuracy of classification [16].

During 2006–2010, the average annual lung cancer incidence rate among United States men ranged from 1.3 cases per 100,000 in age 30–34 years to 585.9 in age 85–89 years. Among women, incidence ranged from 1.4 in age 30–34 years to

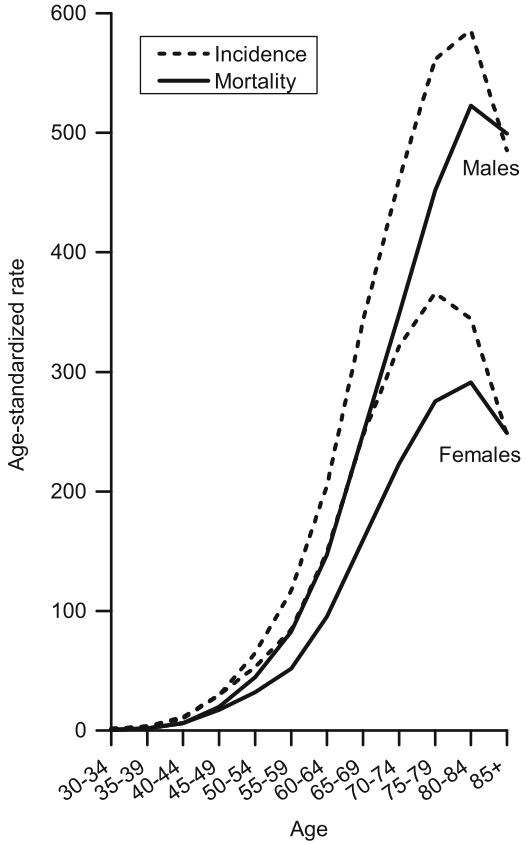

Fig. 1 Lung cancer incidence and mortality rates by sex and age, United States, 2006–2010. Rates are per 100,000 and age-adjusted to the 2000 U.S. standard population

365.8 in age 75–79 years. The median age at diagnosis of lung cancer of men and women combined was about 70 years, with approximately 10 % of cases occurring in those younger than 55 years, 53 % in those 55–74 years, and 37 % in those 75 years and older [17].

Mortality patterns by age closely follow incidence patterns. In 2006–2010, mortality rates among men ranged from 0.6 per 100,000 among those 30–34 years to 522.8 among those 80–84 years. Mortality rates among women ranged from 0.5 per 100,000 among those 30–34 years to 291.2 among those 80–84 years. The median age at death from lung cancer for men and women combined was about 72 years, with about 9 % of deaths occurring among those younger than 55 years, 50 % in those 55–74 years, and 41 % in those 75 years and over [17].

Race/Ethnicity

During 2006–2010, male lung cancer incidence rates were highest among blacks (94.7 per 100,000), followed by non-Hispanic whites (82.9 per 100,000), American Indians/Alaska Natives (70.2 per 100,000), Asians/Pacific Islanders (48.8 per 100,000), and Hispanics (45.9 per 100,000) (Fig. 2). Among women, incidence rates were highest among non-Hispanic Whites (59.9 per 100,000), followed by American Indians/Alaska Natives (52.1 per 100,000), Blacks (50.4 per 100,000), Asians/Pacific Islanders (28.0 per 100,000), and Hispanics (26.6 per 100,000) (Fig. 2). These differences primarily reflect historical smoking patterns. Historically, black men smoked

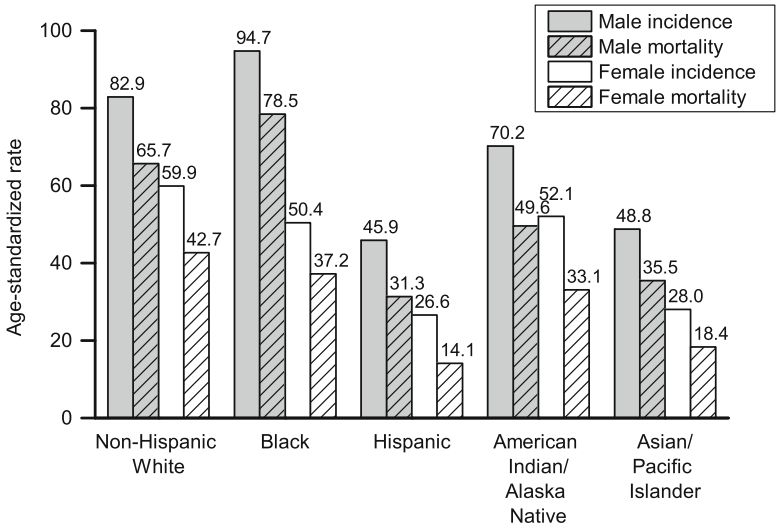

Fig. 2 Lung cancer incidence and mortality rates by sex and race/ethnicity, United States, 2006–2010. Rates are per 100,000 and age-adjusted to the 2000 U.S. standard population. Nonwhite race categories are not mutually exclusive of Hispanic origin (Source: Siegel et al. [2])

at higher rates than white men and men of other racial/ethnic groups. In contrast, black and white women historically smoked at similar rates. However, in the past few decades, black teenagers initiated smoking at lower rates than white teenagers.

Among males, Blacks have the highest mortality rates, followed by non-Hispanic Whites; among females, non-Hispanic Whites have the highest mortality rates, followed by Blacks. Among both males and females, these are followed by American Indians/Alaska Natives, Asians/Pacific Islanders, and Hispanics (Fig. 2). However, it is worth noting that significant heterogeneity in lung cancer rates exist within these broad racial/ethnic groups according to geography and subpopulation. For instance, lung cancer incidence rates in 1999–2004 among American Indian/Alaska Native men range from 22.1 per 100,000 in the Southwest to 116.5 in Alaska. Among women, incidence rates in 1999–2004 range from 10.3 per 100,000 in the Southwest to 97.4 in the Northern Plains [18]. Among Asian Americans, lung cancer incidence rates in 2004–2008 ranged from 30.1 per 100,000 among Asian Indian and Pakistani men to 73.4 among Vietnamese men, while they ranged from 12.1 per 100,000 among Asian Indian and Pakistani women to 31.8 among Vietnamese women [19].

In men, lung cancer mortality rates have been decreasing in all racial/ethnic groups except American Indians/Alaska Natives during the most recent time period (Fig. 4). However, the magnitude of the decreases vary by race/ethnicity. From 2001 to 2010, rates decreased annually by an average of 3.3 % in Black males, 2.8 % in Hispanics, 2.4 % in Whites, and 1.6 % in Asians/Pacific Islanders, while rates were stable in American Indians/Alaska Natives [20]. Among females, lung cancer mortality rates are decreasing among all racial/ethnic groups except American Indians/Alaska Natives and Asians/Pacific Islanders (Fig. 4). From 2001 to 2010, rates decreased annually by an average of −1.1 % in Hispanic females, −1.0 % in Black females, and −0.9 % in Whites, while rates remained stable in American Indians/Alaska Natives and Asians/Pacific Islanders [20].

Sex

Lung cancer mortality rates among females have historically been lower than males, peaking at about 40 deaths per 100,000, or about half of the peak rate of 90 deaths per 100,000 among males (Fig. 3). These patterns are similar when broken down by racial/ethnic group (Fig. 4).

Lung cancer incidence and mortality among males began to increase around the 1920s following the uptake of smoking among men around the turn of the twentieth century [1]. Lung cancer mortality has been decreasing among men since the early 1990s, reflecting widespread smoking cessation that began around 1964 with the release of the United States Surgeon General's report, which concluded that smoking was causally related to lung cancer [21]. Lung cancer incidence and mortality rates among women began to increase in the 1960s (Fig. 3), reflecting the later uptake of smoking among women around the 1930s and 1940s [22]. Smoking cessation among women in the United States began around the 1980s, [22] and mortality rates have been decreasing among women since the 2000s [17].

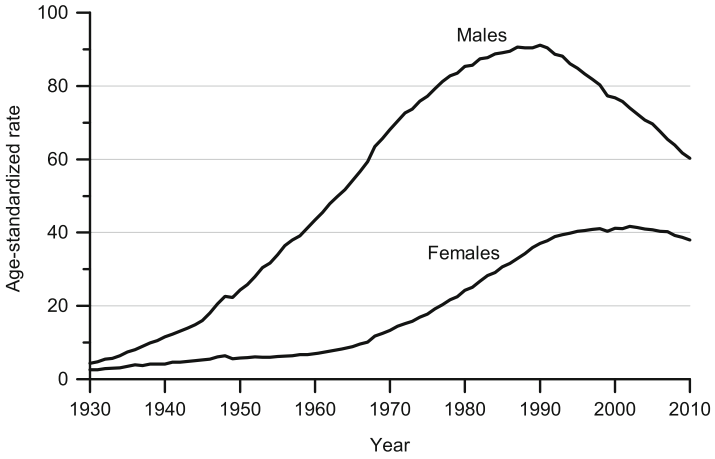

Fig. 3 Lung cancer mortality rates by sex, United States, 1930–2010. Rates are per 100,000 and age-adjusted to the 2000 U.S. standard population. Due to changes in ICD coding, numerator information has changed over time; rates include deaths from lung, bronchus, pleura, trachea, mediastinum, and other respiratory organs (Source: US Mortality Volumes 1930–1959, US Mortality Data 1960–2010, National Center for Health Statistics, Centers for Disease Control and Prevention)

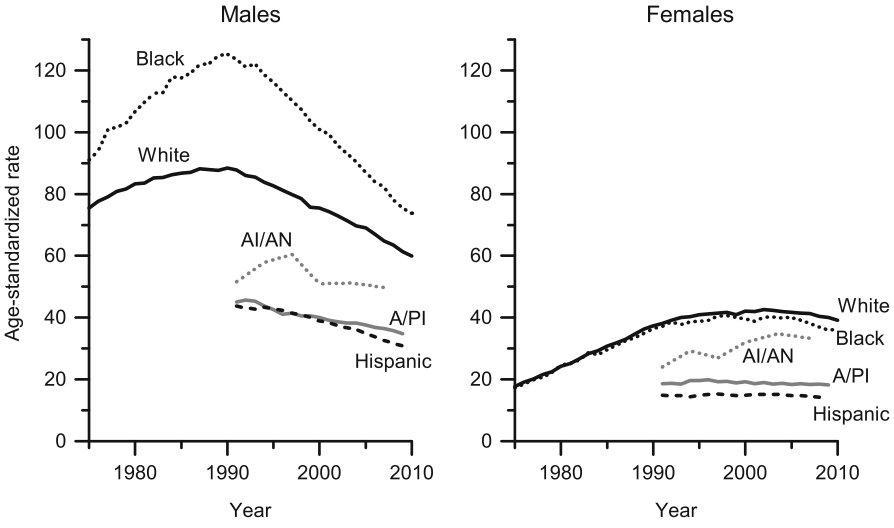

Fig. 4 Lung cancer mortality rates by sex and race/ethnicity, United States, 1975–2010. Rates are per 100,000 and age-adjusted to the 2000 U.S. standard population. Rates for American Indians/Alaska Natives are based on the Contract Health Service Delivery Area counties. Hispanic is not mutually exclusive from whites, blacks, Asian/Pacific Islanders, and American Indians/Alaska Natives. Data for whites rather than non-Hispanic whites is presented because ethnicity data was not available prior to 1990. Mortality data for Hispanics exclude cases from Connecticut, the District of Columbia, Louisiana, Maine, Maryland, Minnesota, Mississippi, New Hampshire, New York, North Dakota, Oklahoma, South Carolina, Vermont, and Virginia. Abbreviations: AI/AN American Indian/Alaska Native; A/PI Asian/Pacific Islander

Socioeconomic Status

Lung cancer rates are primarily linked to socioeconomic status through smoking patterns. In the United States, those with more education and resources are more likely to quit or not initiate smoking [23, 24]. In 2012, smoking prevalence among adults was 32.1 % among those with a 9–11th grade education, 23.1 % among those with a high school education, and 9.1 % among college graduates [25]. The smoking prevalence among those below the poverty threshold was 27.9 %, while the prevalence among those at or above the threshold was 17.0 % [25]. People with a high school diploma or an incomplete high school education are less likely than those with a partial or complete college education to attempt to quit smoking [26].

The higher smoking prevalence among individuals of lower socioeconomic status is reflected in higher lung cancer rates. Among a cohort of US cancer patients diagnosed between 1973 and 2001, the lung cancer incidence rate among men with less than a high school diploma was 166.6 per 100,000, while the rates among high school graduates and college graduates were 123.9 and 57.6, respectively. The relationship was similar among women, with an incidence rate of 71.6 among those with less than a high school diploma, followed by rates of 59.1 among high school graduates and 35.9 among college graduates [27]. In the same cohort, rates also demonstrated a similar trend by family income, ranging from 91.0 per 100,000 among men in the highest income group to 150.9 among men in the lowest income group; rates among women were 45.9 in the highest income group and 81.4 in the lowest income group [27].

Geographic Variation

There is significant regional and state variation in lung cancer rates (Table 1) [13]. Among males, lung cancer mortality rates during 2006–2010 ranged from 27.5 in Utah to 97.1 in Kentucky. Rates were also high in Mississippi (95.4), Arkansas (90.1), Tennessee (89.5), and Alabama (87.4). The lung cancer burden is generally highest in states where tobacco has historically been grown and processed [13]. In addition to Utah, mortality rates were low in New Mexico (43.4), Colorado (44.2), California (47.2), and Hawaii (48.8). Among females, mortality rates during 2006–2010 ranged from 16.8 in Utah to 55.8 in Kentucky. The low rates in Utah can be attributed to the cultural prohibition against smoking among the large Mormon population [13]. Other states with low rates include Hawaii (25.9), New Mexico (28.6), Colorado (31.3), and North Dakota (32.2). Other states with high rates in women were West Virginia (50.9), Delaware (47.7), Indiana (46.7), Oklahoma (46.6), and Tennessee (46.6).

There is substantial variation in lung cancer mortality trends by state relative to the national trends. While the lung cancer death rate has been decreasing among men in the United States, the trends vary by state. For instance, the rates of decrease

Table 1 Lung cancer incidence and mortality rates (per 100,000 population, age-adjusted to the 2000 Standard US population) by sex, race, and state, United States, 2006–2010

	All races						White						Black					
	Both sexes		Males		Females		Both sexes		Males		Females		Both sexes		Males		Females	
State	Incidence	Mortality	Incidence	Mortality	Incidence	Mortality	Incidence	Mortality	Incidence	Mortality	Incidence	Mortality	Incidence	Mortality	Incidence	Mortality	Incidence	Mortality
Alabama	74.9	60.3	103.2	87.4	54	40.8	77.5	62.1	103	86.9	58.2	43.6	65.1	54	105	90.9	39	30.6
Alaska	70.8	52.8	83.8	61.8	60.4	45.8	68.6	50.9	78.3	58.1	60.6	45.2	62.8	51	^	^	^	^
Arizona	54	40.6	61.8	49.8	47.9	33.2	55.3	41.8	63	51	49.1	34.3	53.3	42	59.7	55.2	49.2	32.8
Arkansas†‡	81.1	64.6	108.4	90.1	60.4	45.4	81.6	64.9	107.5	89.1	61.8	46.5	77.8	65.6	117.6	104.8	50.3	39.1
California	51.2	38.7	60.4	47.2	44.4	32.3	52.9	40	60.8	47.6	47	34.4	63.8	50.9	81.1	67.2	51.7	39.3
Colorado	49.2	36.7	56.1	44.2	44.2	31.3	49.4	36.9	56.1	44.1	44.7	31.7	54.9	43.8	65.8	56.5	47.1	34.7
Connecticut	66.1	44.2	75.5	52.7	59.5	38.2	66.9	44.8	75.2	52.7	61.3	39.3	60.3	42	80.5	58.6	46.3	31
Delaware	74.9	56.4	87.1	68.2	65.8	47.7	76.6	57.5	87.9	68.6	68.1	49.2	67.9	53.1	84.4	68.9	56.1	41.6
Washington DC	60.3	46.1	77.5	61.9	48.1	35	39.4	31.4	46.6	37.6	32.8	25.8	71.5	55.3	97	78.6	55.1	40.6
Florida	66.8	48.8	79.4	61.4	56.7	38.8	68.4	50	79.7	61.8	59.3	40.5	53.6	41.1	78.6	63.7	35.8	25.4
Georgia	71	52.9	93.3	73.1	55	38.7	74.6	55.2	94.2	73.7	60.1	41.7	62.5	48.1	94.5	75	42.6	31.8
Hawaii	50	36	64.3	48.8	38.7	25.9	55.1	42.8	62.6	50	48.5	36.4	46.3	39.8	^	^	^	^
Idaho	53.5	41	61.5	49.2	47.2	34.5	53.5	41.3	61.7	49.6	47.2	34.7	^	^	^	^	^	^
Illinois	71.5	51.8	86.7	65.8	60.9	41.9	71.4	51.4	85.4	64.6	61.5	42	81.7	63.2	106.6	86.3	65.5	48.6
Indiana	77.4	60.5	96.4	79.4	63.5	46.7	77.1	60.4	95.6	79	63.7	46.9	84.4	68.1	111.5	94.7	65.6	50.4
Iowa	67.6	49.3	84.7	64.1	54.9	38.3	67.4	49.3	84.4	63.9	54.7	38.4	101.3	71.9	128.4	97.2	82.1	52
Kansas	65.7	51.2	80.2	66.8	54.5	39.4	65.2	50.7	79.4	65.9	54.3	39.1	76.5	64	95.6	86.5	62.8	48.9
Kentucky	99.6	73.2	125.9	97.1	80.3	55.8	99.8	73.2	125.6	96.7	80.7	56	106.8	80.7	144.2	114.1	82.9	59.1
Louisiana	75.7	59.4	99.6	80.9	57.7	43.6	74.9	58.4	94.5	76.1	60	45.3	79.5	63.7	118.4	98.4	53	40.3
Maine	77.5	56.1	91.5	69.9	67.3	46	77.8	56.3	91.9	70.1	67.5	46.2	^	^	^	^	^	^
Maryland	63.5	49.6	74.4	61.7	55.8	40.8	65.7	50.9	74.6	61.3	59.3	43.2	61.1	50.3	78.5	69.6	50.1	37.9
Massachusetts	69.7	49.7	78.3	60.6	64.1	42.1	71.6	51.2	79.4	61.8	66.7	44	49.6	35.3	68.6	52.5	37.2	23.9
Michigan	71.9	54.1	86.1	68.2	61.6	43.9	70.4	53.4	83.3	66.7	61	43.8	82.8	62.4	109.1	85.3	64.8	47

(continued)

Table 1 (continued)

	All races						White						Black					
	Both sexes		Males		Females		Both sexes		Males		Females		Both sexes		Males		Females	
State	Incidence	Mortality	Incidence	Mortality	Incidence	Mortality	Incidence	Mortality	Incidence	Mortality	Incidence	Mortality	Incidence	Mortality	Incidence	Mortality	Incidence	Mortality
Minnesota§	–	43.9	–	53.3	–	37	–	43.9	–	53.1	–	37.3	–	45.6	–	59	–	35.2
Mississippi	80.1	64.3	112.7	95.4	56.2	42	81.3	64.5	109.6	91.7	60.2	44.6	76.7	63.5	120.6	105.3	47	35.8
Missouri	77.5	59	95.6	76.5	64.3	46.2	77	59.1	94.7	76.3	64	46.4	86.6	63.1	108.5	85.6	71.9	48.4
Montana	61.8	45.1	68.9	52.1	56.3	39.6	60.1	44.5	66.9	51.3	54.9	39.1	^	^	^	^	^	^
Nebraska	61.3	46.2	74.6	60.3	51.2	35.5	60.7	46	73.8	60	50.8	35.4	80.2	60.8	97.4	79.4	66.2	46.5
Nevada†¶	69.5	52.4	75.7	59.7	64.7	46.5	73.2	55.5	77.6	61.7	69.9	50.5	54.9	44.1	65.4	51.8	46.2	37.1
New Hampshire	69.8	49.8	80.1	59	62.2	43.3	69.6	50.3	79.6	59.5	62.1	43.7	^	^	^	^	^	^
New Jersey	62.4	44.8	72.8	55.8	55.3	37.2	64.3	46.3	73.8	56.7	58	39.1	62.4	46.3	81.8	65.5	50	34.5
New Mexico	44.7	35.1	52.9	43.4	38.1	28.6	46.5	36.6	54.4	44.8	40.1	30.1	61.5	48.2	89.6	74	35.7	^
New York	64.3	43.5	76.3	54.3	56	36.1	68	46.2	78.5	56.2	60.8	39.1	52.8	36.4	71.3	51	41.5	27.6
North Carolina	73.9	55.7	96.7	76.6	57.2	40.7	74.9	56.4	95	74.7	60	42.9	70.7	54	107.8	89.1	46.5	32.1
North Dakota	53.8	41.5	68.1	54.1	43.3	32.2	52.4	40.4	66.9	53.4	41.5	30.7	^	^	^	^	^	^
Ohio†¶	74.2	57.1	92.6	74.8	60.7	44.2	73.4	56.6	91.1	73.7	60.3	44	84	66.4	111.3	92.8	66.1	49.2
Oklahoma	77.1	60.8	96.1	79.6	62.7	46.6	75.2	61.4	93.4	79.8	61.5	47.4	76.7	59.1	106.2	82.2	54.8	42
Oregon	63.2	49.5	70.6	58.4	57.6	42.7	62.9	50	69.8	58.9	57.7	43.3	72.2	59.9	93.2	79.8	53.2	42.7
Pennsylvania	68.9	50.5	84.4	65.8	57.9	39.6	67.9	49.9	83.1	65	56.9	39.1	85.7	63.3	107.2	84.9	72.5	50.2
Rhode Island	72.1	51.4	84.1	64.9	64.5	42.6	73.2	52.2	85.5	65.8	65.3	43.4	60.7	43.9	59.7	52.9	62.9	37.3
South Carolina	71	55.6	94.1	77.1	53.9	39.9	73.4	56.9	93.3	75.7	58.3	42.8	62.7	51.3	96.5	83.2	40.1	30.6
South Dakota	59.1	46	73.8	61.9	48	34.1	57.9	45.3	72.6	60.8	46.7	33.8	^	^	^	^	^	^
Tennessee	79.1	64.5	103.4	89.5	61.3	46.6	79.8	64.7	103.3	88.7	62.5	47.3	75.5	65.9	105.5	100.8	55.7	44

Texas	61.6	46.2	78.2	60.7	49	35.4	61.1	45.8	76.6	59.2	49.4	35.7	73.6	58.2	106.4	88.7	51.5	37.9
Utah	28.1	21.6	34.1	27.5	23.3	16.8	28	21.4	34	27.3	23.1	16.7	55.5	53.4	^	^	^	^
Vermont	72.2	52.5	81.2	62.3	65.6	45.1	72.5	52.8	81.5	62.9	65.9	45.2	^	^	^	^	^	^
Virginia†	65.9	51.4	82.2	67.1	53.9	40	66.7	51.7	81	65.7	56	41.4	68.6	55.8	97.6	83.2	49.5	38.4
Washington	63.5	48.5	72.1	57	57.3	42.1	64.4	49.5	72.1	57.6	58.7	43.5	71	53.2	87.1	65.7	57.8	42.7
West Virginia	85.7	65.2	106.4	84.3	70	50.9	86.1	65.6	106.7	84.7	70.6	51.4	78.3	61.2	109.4	85.4	54.1	42.7
Wisconsin	62	46.9	73.6	58.4	53.4	38.4	59.3	46	69.9	57	51.6	38	90.4	70.4	123.9	103.4	67.2	47.7
Wyoming	51.1	42.4	57.3	49.9	46	36.4	51	42.6	57.3	50.4	45.8	36.3	^	^	^	^	^	^

Sources

Incidence: NAACCR, 2013. Data are collected by cancer registries participating in the National Cancer Institute's SEER program and the Centers for Disease Control and Prevention's National Program of Cancer Registries

Mortality: US Mortality Data, National Center for Health Statistics, Centers for Disease Control and Prevention

^ Statistic not displayed due to fewer than 25 cases

† This state's data are not included in US combined rates because they did not meet high-quality standards for one or more years during 2006–2010 according to the North American Association of Central Cancer Registries (NAACCR)

‡ Rates are based on incidence data for 2006–2008

§ This state's registry did not submit 2006–2010 cancer incidence data to NAACCR

¶ Rates are based on incidence data for 2006–2009

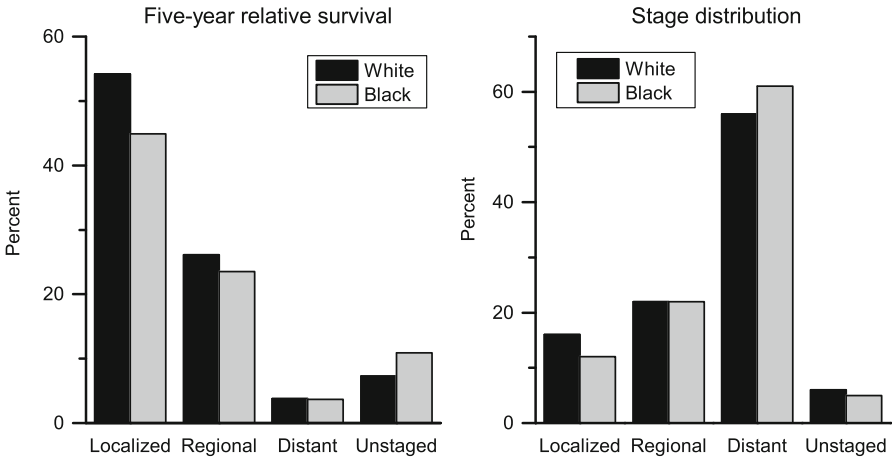

Fig. 5 Lung cancer 5 year relative survival and stage distribution, United States, 2003–2009 (Source: Howlader [17])

in California, which was the first state to enact comprehensive tobacco control measures, are nearly twice that of many states in the Midwest and South [13]. Among females, while overall national lung cancer death rates have leveled off and begun to decrease, rates in several states in the Midwest and South continue to increase [13, 28]. These states are characterized by low excise taxes on cigarettes and lack of other tobacco control policies [28].

Survival

Lung cancer survival is low and has seen only marginal increases since the mid-1970s [17]. Based on data from the nine oldest SEER registries, the 5 year relative survival rate increased from 12 % for patients diagnosed during 1975–1977 to 18 % for those diagnosed during 2003–2009 [17].

Cancer survival depends largely on stage at diagnosis (Fig. 5). For lung cancer patients diagnosed in 2003–2009, the 5 year relative survival rate was 54 % for localized stage disease, 26 % for regional stage, and 4 % for distant stage [17]. However, only 15 % of cases were diagnosed at the localized stage, while 22 % were diagnosed at the regional stage and 57 % were diagnosed at the distant stage [17].

Survival is lower in blacks (14 %) than in whites (18 %) [17] because blacks are less likely to receive standard treatment and are more likely to be diagnosed at an advanced stage (Fig. 5). Survival also declines with age. The 5 year relative survival rate for those diagnosed before the age of 45 is 27 %, compared to 19 % among those diagnosed at ages 55–64 and 12 % among those diagnosed at age 75 or greater [17].

Global Lung Cancer Patterns

Worldwide, lung cancer is the leading cause of cancer death in men and the second leading cause of cancer death in women, with approximately 1.8 million new cases and 1.6 million deaths annually [3]. Across the world, the lung cancer burden varies substantially across countries and regions.

Global Variations in Incidence and Mortality

The significant worldwide variation in lung cancer rates largely reflects differences in the stage and degree of the tobacco epidemic, though differences in air pollution are also a factor. Among males, the highest incidence and mortality rates occur in Central, Eastern, Southern, and Western Europe, Northern America, Micronesia/Polynesia, and Eastern Asia (Figs. 6 and 7), whereas the lowest rates occur in Middle, Western, and Eastern Africa. Overall, incidence rates range from 1.7 per 100,000 in Western Africa to 53.5 in Central and Eastern Europe, while mortality rates range from 1.5 in Western Africa to 47.6 in Central and Eastern Europe [3].

In men, lung cancer incidence rates are about 50 % higher in more-developed regions (44.7 cases per 100,000) compared to less-developed regions (30.0 per 100,000) [3]. However, due to their larger populations, less-developed regions accounted for about 60 % (1.1 million out of 1.8 million) of all lung cancer cases in 2012 [3]. The lung cancer burden among males is predominantly shaped by smoking

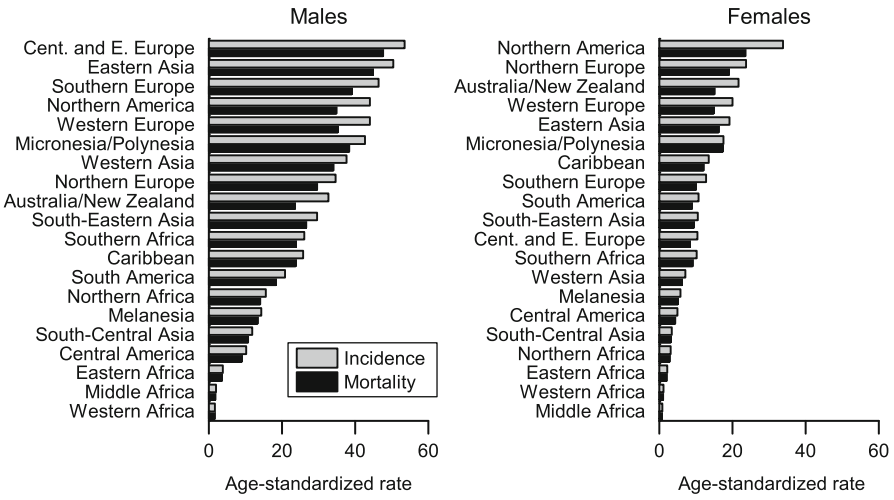

Fig. 6 Lung cancer incidence and mortality rates by sex and world region, 2012. Rates are per 100,000 and age-adjusted to the 1960 world standard population (Source: Ferlay [3]. Accessed on 12/13/2013)

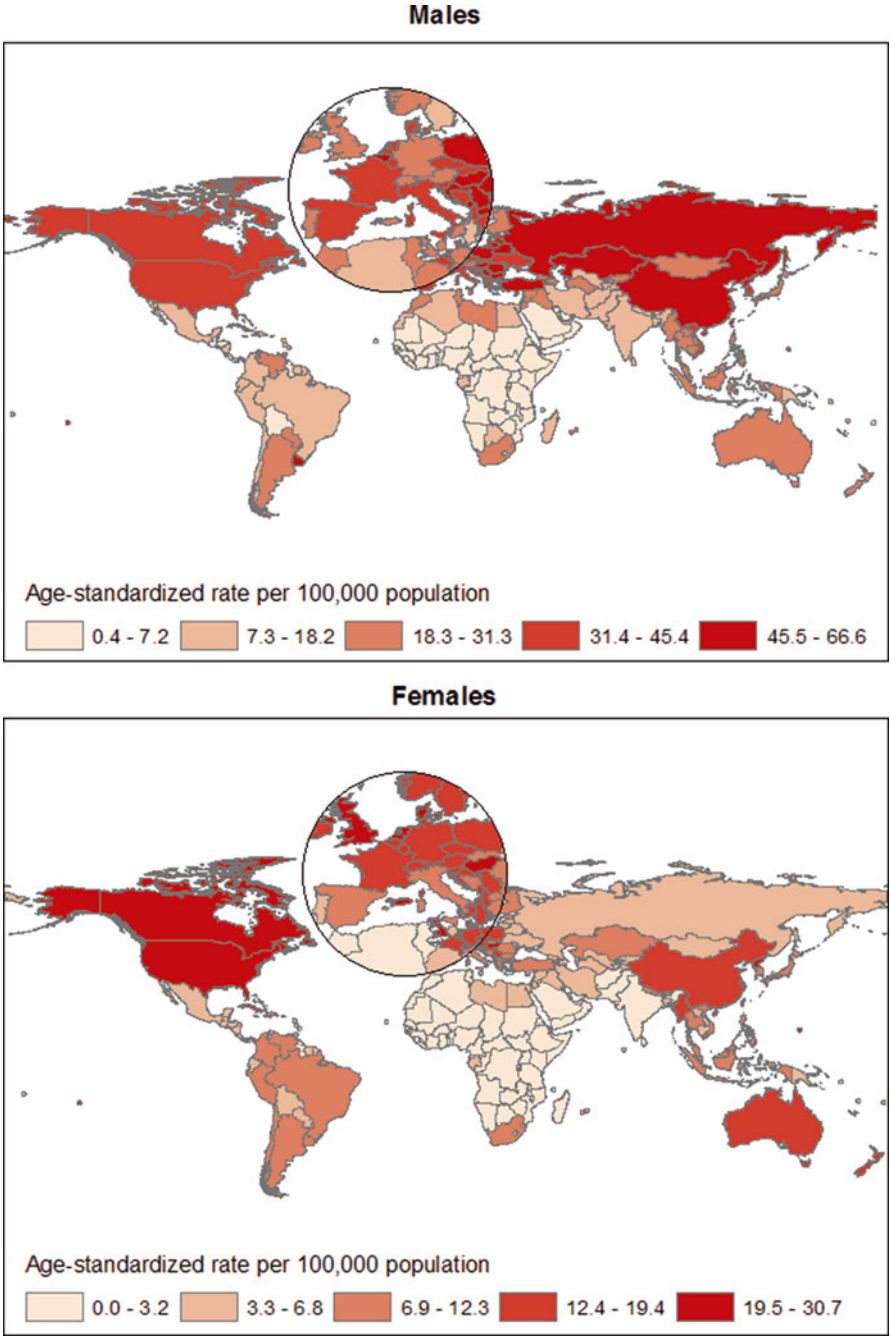

Fig. 7 International variation in lung cancer mortality by sex, 2012. Rates are per 100,000 and age-adjusted to the 1960 world standard population (Source: Ferlay [3]. Accessed on 3/5/2014)

patterns, although other factors, such as air pollution and occupational exposures, also play a role [29].

Among females, the highest incidence and mortality rates occur in Northern America, Northern and Western Europe, Australia/New Zealand, and Eastern Asia (Figs. 6 and 7). The lowest rates occur in Middle, Western, Eastern, and Northern Africa. Overall, incidence rates among females range from 0.8 per 100,000 in Middle Africa to 33.8 in Northern America, while mortality rates range from 0.7 in Middle Africa to 23.5 in Northern America [3]. By country, mortality rates vary from 0.0 in Comoros, Samoa, and Niger to 30.7 in North Korea [3]. The lung cancer burden among females, while largely linked to smoking patterns, is also related to other risk factors including air pollution and occupational exposures [29]. In Eastern Asia in particular, where smoking among women remains uncommon, indoor air pollution from cooking and heating plays a significant role [29, 30].

Variation in the lung cancer burden exists not only across regions, but also across countries and even within each country. For example, in Africa, male lung cancer incidence rates range from 0.4 per 100,000 in Niger to 32.3 in La Reunion. In many countries, there is also wide variation in lung cancer rates within the country [3]. For instance, in Singapore among men, lung cancer rates for the Indian population are 17.4 per 100,000, compared to 34.0 in the Malay population and 44.7 in the Chinese population [10]. In females, variation within regions and within countries is also notable. For example, in Eastern Europe, incidence rates range from 6.1 per 100,000 in Ukraine to 33.2 in Hungary [3]. In New Zealand, lung cancer incidence is 25.0 per 100,000 among Pacific Islanders and 79.0 among the Maori population [10].

Survival

Survival for lung cancer is poor and does not vary a great deal between high-income and low- and middle-income countries, although high-income countries may have slightly better survival rates due to improved detection and access to treatment. For example, 5 year relative survival for lung cancer is 7 % in India and 9 % in Thailand, compared to 17 % in Australia and 18 % in Canada [31].

Global Trends in Incidence and Mortality

In countries where smoking uptake began earliest, such as Canada, the United States, United Kingdom, and Australia, lung cancer incidence and mortality rates among males have been declining since the 1970s–1990s [12]. Rates among males are now also declining in most countries of Europe and North America, as well as select countries in South America (Argentina, Chile, Costa Rica, Mexico) and high-income populations of Asia, such as Hong Kong, Singapore, Japan, and South Korea [12]. In contrast, in countries where the smoking epidemic began more

recently, including low- and middle-income countries of South America and Asia, lung cancer mortality rates continue to rise [12]. Little incidence and mortality data exists for Africa, but evidence suggests that smoking is becoming more prevalent among males in many countries in Sub-Saharan Africa, [32] which could lead to increasing lung cancer rates in the future.

Lung cancer trends among women differ from those among men due to a different progression of smoking uptake. In countries where smoking uptake among women began earlier, lung cancer incidence and mortality rates are approaching a peak or have peaked in recent years. For instance, lung cancer mortality rates among adult women aged 30–74 in Denmark and the United States have been decreasing since 1995 and 1992 respectively, and rates in Canada have been stable since 1996 [33]. In other countries where the tobacco epidemic began later, especially in Western and Southern Europe and most countries of Eastern Europe and South America, rates continue to increase [33]. In many low- and middle-income countries where the tobacco epidemic has not yet begun, limited evidence indicates that lung cancer rates have remained low. For these countries, however, data is scarce, and detecting lung cancer trends may be difficult. Smoking among women is on the rise in many countries where it was previously rare, such as Russia and Ukraine, which will likely lead to increasing lung cancer rates in the future [34, 35].

Summary

Lung cancer is the leading cause of cancer death in the United States in both men and women. Patterns are primarily driven by smoking. Rates are highest in males, people of lower socioeconomic status, and in certain states of the South and Midwest. Lung cancer incidence and mortality rates at the national level are decreasing among both males and females, although trends are not equal across demographic or geographic groups. For instance, lung cancer mortality rates continue to rise among females in select states of the South and Midwest. As people of higher socioeconomic status are now less likely to initiate smoking and are more likely to quit, the burden of lung cancer in the United States also falls increasingly to those of lower socioeconomic status.

Worldwide, lung cancer is the leading cause of death among men and the second leading cause of death among women, after breast cancer. Lung cancer incidence and mortality rates are highest in high-income countries such as those in Europe, North America, and Oceania where smoking uptake occurred earliest, although rates are now decreasing in many of these countries. Rates are also high in Eastern Asia, driven by a rapid uptake of smoking among males and exposure to indoor air pollution among females. Rates continue to increase in many low- and middle-income countries where smoking uptake occurred later. Lung cancer deaths can be averted through tobacco control measures aimed at prevention of smoking initiation as well as smoking cessation.

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Epidemiology of Lung Cancer

Ann G. Schwartz and Michele L. Cote

Abstract Lung cancer continues to be one of the most common causes of cancer death despite understanding the major cause of the disease: cigarette smoking. Smoking increases lung cancer risk 5- to 10-fold with a clear dose–response relationship. Exposure to environmental tobacco smoke among nonsmokers increases lung cancer risk about 20 %. Risks for marijuana and hookah use, and the new e-cigarettes, are yet to be consistently defined and will be important areas for continued research as use of these products increases. Other known environmental risk factors include exposures to radon, asbestos, diesel, and ionizing radiation. Host factors have also been associated with lung cancer risk, including family history of lung cancer, history of chronic obstructive pulmonary disease and infections. Studies to identify genes associated with lung cancer susceptibility have consistently identified chromosomal regions on 15q25, 6p21 and 5p15 associated with lung cancer risk. Risk prediction models for lung cancer typically include age, sex, cigarette smoking intensity and/or duration, medical history, and occupational exposures, however there is not yet a risk prediction model currently recommended for general use. As lung cancer screening becomes more widespread, a validated model will be needed to better define risk groups to inform screening guidelines.

Keywords Lung cancer • Epidemiology • Smoking • Genetics • Susceptibility • Risk models

As discussed in chapter "Lung Cancer Statistics", lung cancer is the most common cause of cancer death in the United States, is the second most frequent cancer diagnosed, behind breast cancer in women and prostate cancer in men, and is one of the few cancers with a strong environmental exposure definitively linked to risk. It is also a cancer for which little progress has been made in terms of early detection and survival. Eighty to ninety percent of all lung cancers are attributable to cigarette smoking and could be prevented. While both lung cancer incidence and mortality

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rates have fallen with the reduction in tobacco smoking, lung cancer continues to be the cause of significant morbidity and mortality. This chapter will present the epidemiology of lung cancer, including both well studied risk factors, such as cigarette smoking, and other risk factors such as family history and genetic susceptibility, that are often overlooked because of the strength of the association with smoking.

Smoking

In 1964, the landmark report on smoking and health was released by the Surgeon General of the U.S. Public Health Service. The report detailed the association between lung cancer and cigarette smoking, noting that men who were average smokers had a 9 to 10-fold greater risk of lung cancer than nonsmoking men, and that risk estimates were even higher for heavy smokers [1]. In the decades prior to the report, smoking was prevalent in the U.S., with two thirds of adult men and one third of adult women reporting current smoking in 1955. 2014 was the 50th anniversary of the report, and as a whole the U.S. has made great strides in reducing cigarette smoking.

In 2010, 19.3 % of U.S. adults reported current smoking, although the declines have not been equal across population subgroups [2]. Current smoking is more prevalent among men (21.2 %) compared to women (17.5 %). Race and ethnicity also influences cigarette smoking, with highest rates among American Indian or Alaska Natives (26.6 %) and lowest rates among Asians (9.3 %). Current smoking prevalence by race/ethnicity and gender is shown in Fig. 1 for the most common race/ethnic groups in the U.S. Age is also associated with current smoking status,

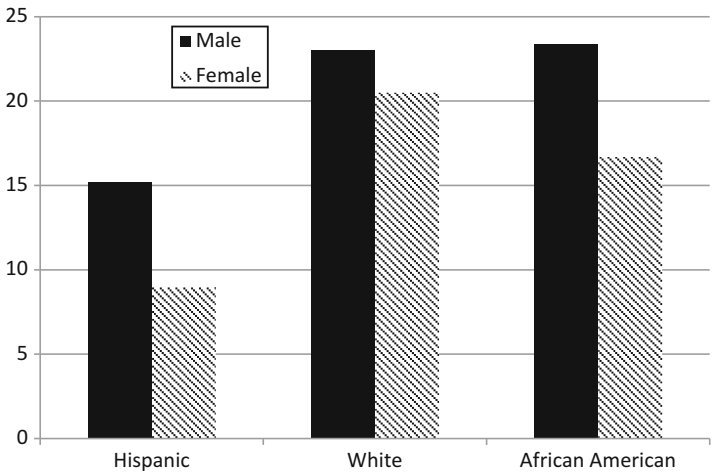

Fig. 1 Prevalence of current cigarette smoking, by race/ethnicity and sex, 2010, National Health Interview Survey, United States [2]

with only 5.1 % of individuals ages 75 and older reporting smoking, compared to 21.5 % of those ages 18–44 years. Smoking is more prevalent among those who live below the poverty level (28.4 %) and those without a high school diploma (27.1 %). There are regional differences as well, with those living in the Midwest and South more likely to smoke (21.8 % and 21.0 %, respectively) compared to those living in the Northeast (17.4 %) or West (15.9 %). These data are from the 2010 U.S. National Health Interview Survey, a household survey conducted annually by interviewers of the U.S. Census Bureau for the Centers for Disease Control and Prevention (CDC) [2].

While personal cigarette smoking has been causally linked with lung cancer, second-hand cigarette smoking, also called involuntary smoking or environmental tobacco smoke (ETS), has been associated with lung cancer risk in exposed nonsmoking individuals. In 1986, the Surgeon General released a report detailing the chemical composition of sidestream smoke, noting it is qualitatively similar to the mainstream smoke inhaled by the smoker and that both mainstream and sidestream smoke act as carcinogens [3]. This report also concluded ETS exposure is associated with lung cancer in nonsmoking individuals. Various epidemiologic studies have confirmed the association with lung cancer, although the findings for ETS are neither as strong nor as consistent as the risk estimates reported for current smoking. This is not unexpected, as measuring ETS exposure is less standardized than estimating years smoked, or number of cigarettes used per day. Despite difficulties with assessing ETS exposure, most epidemiologic evidence supports a modest association between ETS exposure and lung cancer. A meta-analysis of 35 case–control and 5 cohort studies suggested nonsmoking women exposed to ETS from their spouse's smoking had a 1.2-fold increase in risk compared to women who were not exposed (OR = 1.20, 95 % CI: 1.10–1.29) [4]. Since personal smoking is more prevalent among men, similar studies estimating risk of lung cancer among men from exposure to ETS through their smoking spouse are not available. For workplace ETS, another meta-analysis of 22 workplace studies found a 24 % increase in risk of lung cancer among workers exposed to ETS (RR = 1.24, 95 % CI: 1.18, 1.29) [5]. Longer durations of exposure, whether in the workplace or at home, have been associated with even greater increases in risk.

Other methods to smoke tobacco, such as pipe and cigar smoking, are also associated with increased risk of lung cancer. Two studies from the American Cancer Society's Cancer Prevention Study cohort provide estimates of risk for men; however, data for women are not available because of low usage of these products among women. Men who reported current or former exclusive pipe smoking (i.e., did not also smoke cigarettes) had a 5-fold increased risk of death from lung cancer compared to men who reported never using tobacco (including cigarettes) (RR = 5.00, 95 % CI 4.16, 6.01) [6]. In this same cohort, men who reported current exclusive cigar smoking at baseline had an nearly identical risk of dying from lung cancer compared to those who never used tobacco (RR = 5.1, 95 % CI: 4.0–6.6) [7]. A cohort study from Europe of 102,395 men reported slightly lower estimates for exclusive users of pipes (HR = 3.0, 95 % CI: 2.1, 4.5) or cigars (HR = 2.2, 95 % CI: 1.3, 3.8) compared to men who did not use any tobacco [8]. While these estimates are lower than for cigarette smoking, it should be noted that pipe or cigar smoking

is not a safer alternative to cigarette smoking, but the lower risk is likely explained by lower smoking intensity and perhaps lesser degrees of inhalation of these products.

Other products are also potential risk factors for lung cancer that will likely generate more research in the upcoming decades. First, marijuana (cannabis) use is reported to be the most widely consumed illicit drug worldwide, and the smoke contains many of the same carcinogens found in tobacco. In the U.S., marijuana use has been decriminalized for medical purposes in some regions and to a lesser extent, for personal use. The long-term effects of marijuana use on lung cancer are largely unknown. A 40-year cohort study of 49,321 Swedish men enrolled between the ages of 18 and 20 suggests that "heavy" use of marijuana smoking (defined as having used more than 50 times prior to enrollment) was associated with a 2-fold increase in risk of lung cancer (HR = 2.12, 95 % CI: 1.08, 4.14) after adjustment for tobacco use at baseline and other potential confounders [9]. Unfortunately, these data were only collected at baseline, so updated exposure status could not be incorporated into the models. Marijuana use is prevalent among youth in the U.S., with data from 2011 suggesting that 39.9 % of high school students (9th–12th grades) had tried marijuana at least once, and 23.1 % had used in the 30 days prior to the survey [10]. Thus, marijuana is poised to become an increasingly important risk factor for lung cancer.

Another potential factor for lung cancer is hookah use (water pipe tobacco smoking). Research suggests that a single session of hookah use results in similar mean peak plasma nicotine concentration levels compared to smoking a cigarette, but has 3.75-fold greater carboxyhemoglobin (COHb) levels, and 56-fold greater inhaled smoke volume [11]. The tobacco products used in hookah pipes are often enhanced with various flavorings, and the potential health effects of these chemicals have not been studied. The Monitoring the Future survey found that in 2011, 18.5 % of 12th grade students in the United States had used hookahs in the past year [12]. Other studies indicate that hookah smoking is more prevalent among university students in the United States, with past-year use ranging from 22 % to 40 %, and note that hookah users are more likely to use cigarettes and marijuana [13, 14]. Lastly, electronic cigarettes (e-cigs) or "vapors" are also emerging as an alternative way to inhale nicotine, although little research has been done on these products. It will be necessary for future studies of lung cancer risk to include comprehensive exposure questionnaires to account for various routes of inhaled nicotine, tobacco, and marijuana exposure.

Environmental Exposures

Compared to cigarette smoking, the proportion of lung cancers associated with environmental or occupational exposures is relatively low in the United States, but of significant concern to the 10–15 % of never smokers who develop lung cancer, and also because many environmental exposures may act synergistically with cigarette

smoking. Many agents have been examined as potential risk factors associated with lung cancer but are often difficult to quantify and thus the evidence is unclear. Below we describe exposures which have been linked to lung cancer and are of particular interest due to their ubiquitous nature: radon, air pollution, asbestos, diesel exhaust, and ionizing radiation.

Radon is widely accepted as the first identified environmental cause of lung cancer in studies of underground miners (1920s). An inert gas, it is naturally produced from radium in the decay series of uranium (found in rocks and soil) and is a ubiquitous contaminant of indoor air. A meta-analysis of 13 European case–control studies suggests risk of lung cancer is increased by 8.4 % (95 % CI: 3.0–15.8) per 100 Becquerels/m^3 increase in measured radon (p-value = 0.0007) and they noted a linear dose–response relationship. There was a synergistic effect with current cigarette smoking, with absolute risk at least 25 times greater for smokers [15, 16]. A pooled analysis of over 4000 cases and 5000 controls from 7 North American case–control studies of lung cancer reported similar findings [17]. Estimates suggest that 20,000 lung cancers diagnosed annually in the United States are attributed to radon exposure [18]. As radon is odorless and colorless, most people are unaware of this potential household hazard. Thus, in 2011, 10 United States federal agencies, led by the Environmental Protection Agency, developed a plan to increase awareness and to reduce the risk from radon exposure [19].

Radon can be considered indoor air pollution, as can ETS, but there are additional indoor contaminates that may increase lung cancer risk. In particular, the use of soft coal for cooking and heating has been associated with lung cancer. A meta-analysis of 25 case–control studies with over 10,000 cases and 13,000 controls noted that household coal use was associated with lung cancer in all studies (meta-OR = 2.15, 95 % CI: 1.61–2.89), and stronger associations were seen in studies from China [20]. Several studies of Chinese nonsmoking women report that heating cooking oils to high temperatures is associated with increased risk of lung cancer [21, 22]. The combination of burning coal, cooking fumes, and ETS exposure play an important role in the development of lung cancer for this population. Outdoors, long-term ambient fine particulate matter ($PM_{2.5}$) air pollution has been studied as a potential risk factor for lung cancer, usually through cohort studies that link with air monitoring networks. These studies have shown increased risk of lung cancer mortality as $PM_{2.5}$ levels rise, among individuals living in these areas longer-term [23, 24]. These associations are small, and may be confounded by other exposures, such as cigarette smoking. Regardless of the source, air pollution is a source of concern and continual study for lung cancer and other pulmonary conditions is needed.

A well-established occupational risk factor for lung cancer is asbestos. Asbestos refers to naturally occurring silicate mineral fibers, which have been widely used in industry. Asbestos exposure is related to both mesothelioma and lung cancer, responsible for a combined 10,000 deaths annually in the United States [25]. Asbestos-induced effects in the lungs appear to be dose-dependent and related to the size and composition of the fiber inhaled, with effect sizes ranging from OR = 2.0 to 6.0, depending on the fiber type [25, 26]. There also appears to be a synergistic relationship between cigarette smoking and asbestos exposure, highlighting the need for smoking prevention and cessation for workers in this industry [27].

Evidence supporting increased lung cancer risk with occupational diesel exposure is less established, but a pooled analysis of 11 case–control studies suggested about a 30 % increase in risk among the exposed (OR = 1.3, 95 % CI: 1.2, 1.4) and a significant dose–response trend [28]. A recent review of various studies and exposure assessment methods argues that evidence is still insufficient to make this claim, although the particles found in diesel exhaust contain known carcinogens [29]. In addition, millions of people living in urban areas are exposed to various levels of diesel across their lifespans, and little is known about lung cancer risk associated with low level, chronic exposure.

Lastly, another common exposure that may increase risk of lung cancer is ionizing radiation. Studies of the Hiroshima and Nagasaki atomic bomb survivors suggest increased lung cancer incidence (as well as other solid tumors) among those exposed, with risk increasing in a linear dose response pattern [30]. This single, high dose exposure differs from the smaller doses the general population may receive during X-ray or computed tomography (CT) screenings [31]. Risks associated with repeated CT screenings, while relatively low for an individual, have been considered when making recommendations for implementing population-based lung cancer screening, so that the increased risk of screening does not outweigh the potential benefit [32].

Family History of Lung Cancer

Epidemiologic evidence demonstrates familial aggregation of lung cancer after adjusting for familial clustering of cigarette smoking and other risk factors. Familial aggregation of lung cancer was first noted 50 years ago by Tokuhata and Lilienfeld [33, 34]. In a study of 270 lung cancer patients and 270 matched controls, and their relatives, they found 2.0 to 2.5-fold increased lung cancer mortality in smoking relatives of cases as compared with smoking relatives of controls. A similar finding was noted in nonsmoking relatives. There was an interaction between family history and smoking, with smoking relatives of lung cancer patients having a higher risk of lung cancer than either nonsmoking relatives of lung cancer patients or smoking relatives of controls. This was the first study to account for age and smoking status in a study of familial aggregation of lung cancer, however, smoking intensity or duration was not available.

Several other studies have since reported familial aggregation of lung cancer [35, 36], with the best studies taking into account the number of relatives in the families and the risk factor profiles for each relative to ensure that clustering of smoking habits is not driving aggregation of lung cancer. Studies in southern Louisiana, Houston, Detroit and Iceland reported an increased familial risk of lung cancer among relatives of lung cancer probands (the index case leading the family to be studied) after accounting for the effects of age, sex, and smoking history, and occupation or history of COPD [37–41]. These studies suggested a 2 to 4-fold increased risk associated with having a first degree relative with lung cancer after accounting

for risk factors, including smoking amount and duration, among the relatives, with variation in risk estimates by age of the proband, smoking status and race.

While the studies described above included risk factor data among relatives, pooled and meta-analyses have been conducted that include a broader range of studies. A meta-analysis of 28 case–control studies and 17 cohort studies demonstrated fairly consistent findings of an approximately 2-fold increased risk of lung cancer associated with family history [35]. Risk was generally higher in relatives of cases diagnosed at a young age and when multiple family members were affected. The International Lung Cancer Consortium study included data from approximately 24,000 lung cancer cases and 23,000 controls and reported a significant 1.5-fold increased risk of lung cancer associated with family history after adjustment for smoking and other potential confounders in cases and controls, and a significant 1.3-fold increased risk for lung cancer among never smokers [36]. Risk estimates were similar when evaluating only those studies with risk factor data for each family member; relative risks for lung cancer among relatives with a family history were 1.6 overall, 1.5 for white, 2.1 for African American, and 2.0 for early-onset (<age 50) case relatives. These studies provide substantial evidence for familial aggregation of lung cancer that remains after adjustment for clustering of cigarette smoking within family members.

Genetic Susceptibility

Evidence of familial aggregation of lung cancer suggests that there is a genetic contribution to lung cancer susceptibility, and typically suggests a rare, highly penetrant inherited mutation. In addition, smokers have differential susceptibility to lung carcinogens; only 15 % of smokers develop lung cancer and 10–15 % of lung cancers develop in never smokers. It is possible that variation in genetic profiles contributes to this differential susceptibility, most likely in the form of a more common, low penetrant genetic alteration.

Rare, High Penetrance Genes Only one large, family-based lung cancer study has been conducted providing the first evidence of a lung cancer susceptibility locus on chromosome 6 [42]. In this consortium study, multipoint parametric linkage under the simple dominant low-penetrance affected only model yielded a maximum heterogeneity LOD (HLOD) score of 2.79 at 155 cM (marker D6S2436) on chromosome 6q23-25, with 67 % of the families estimated to be linked. Higher HLODs at this location were reported for more highly affected families: families with four affected relatives gave an HLOD of 3.47, families with five or more affected members in two or more generations, gave an HLOD was 4.26, with 94 % of the families estimated to be linked to this region. In expanded analyses with additional families, the region on 6q was again identified [43]. In addition, lung cancer risk among putative carriers was estimated and found to be higher than among noncarriers, even among never smokers. The usual dose response curves of increasing lung cancer

risk with increasing amount smoked was demonstrated among smoking noncarriers. Among smoking carriers, risk was higher than among noncarriers, but a dose response relationship was not apparent suggesting that any level of tobacco exposure increases risk among those with inherited lung cancer susceptibility. Additional evidence suggestive for linkage was also found for regions on chromosomes 1q, 8q, 9p, 12q, 5q, 14q and 16q [43, 44].

Common, Low Penetrance Genes Initial studies designed to identify more common, low penetrance genes with more moderate effects evaluated small numbers of genetic polymorphisms in biologically plausible pathways including metabolic genes, growth factors, growth factor receptors, DNA damage and repair genes, oncogenes and tumor suppressor genes [45, 46]. More recently, genome-wide association studies (GWAS) have been conducted that rely on very large samples and more than 300,000 markers across the genome. Unlike the candidate gene studies, the GWAS have provided highly significant and reproducible results.

The first three publications of lung cancer GWAS findings identified the same region of chromosome 15q as significantly associated with lung cancer risk [47–49]. This region includes a neuronal nicotinic acetylcholine receptor gene cluster comprising *CHRNA3, CHRNA5* and *CHRNA4* subunits. Genetic variation in this 15q25 region was associated with an approximately 1.3-fold increased risk of lung cancer among individuals carrying a heterozygous mutation (44.2 % of controls for marker rs8034191) and about a 1.8-fold increase for individuals homozygous for the mutation (10.7 % of controls). This region has also been associated with smoking behavior. One study suggested that the region affected smoking behavior [48], another found stronger effects on lung cancer risk that remained after adjusting for smoking behavior [49], while the third study did not find any association with smoking behaviors [47]. A meta-analysis of smokers, lung cancer cases and lung cancer-free controls, and chronic obstructive pulmonary disease (COPD) cases and COPD-free controls reported that multiple loci within this region are associated with cigarettes smoked per day and at least one locus associated with lung cancer independent of amount smoked [50].

Two other regions, on chromosomes 6p21 and 5p15, identified from GWAS have been consistently associated with lung cancer risk [47–49, 51, 52]. *BAT3* and *MSH5* are located in the 6p21 region, while *TERT* and *CLPTM1L* are located in the 5p15 region. In a large meta-analysis of 14,900 lung cancer cases and 29,485 controls from 16 GWAS, all of European ancestry (as were the initial GWAS), additional support was provided for loci associated with increased lung cancer risk at 5p15, 6p21, and 15q25 [53]. Lung cancer GWAS have also been conducted in the Han Chinese population where evidence was found for lung cancer risk associations at 5p15, 3q28 (*TP63*), 13q12 (*MIPEP-TNFRSF19*), and 22q12 (*MTMR3-HORMAD2-LIF*) [54], and at 10p14, 5q32 and 20q13 [55]. In the Japanese population, the findings on 5p15, 3q28, and 6p21 were replicated [56].

Genetic susceptibility for lung cancer in never smokers is less well studied due to the smaller number of never smokers with lung cancer. In a GWAS of never smoking women in Asia, the 6p21, 5p15 and 3q28 findings were replicated and new

regions on 10q25 and 6q22 were identified as being associated with lung cancer [57]. A large GWAS in European American never smokers is underway. In addition, a lung cancer GWAS in African Americans is being conducted. While the findings from the GWAS in African Americans have yet to be published, associations between lung cancer risk and SNPs on 15q25, 5p15 and 6p21 have been replicated in African Americans [58, 59]. A GWAS in lung cancer cases with a strong family history of lung cancer has also been conducted, but results have yet to be published. GWAS in various population subsets who have different genetic backgrounds and smoking behaviors will provide important information for the eventual identification of lung cancer susceptibility genes.

Chronic Obstructive Pulmonary Disease (COPD)

COPD and lung cancer share a common risk factor, cigarette smoking, but studies also suggest that COPD itself is a risk factor for lung cancer independent of smoking habits. A COPD diagnosis has been consistently reported to be associated with a 2- to 3-fold risk of developing lung cancer [60–66], even among never smokers [67]. Lung cancer risk varies with specific COPD phenotypes, i.e., emphysema and chronic bronchitis [62, 66, 68–72]. In a meta-analysis, lung cancer was associated with a previous history of COPD (OR = 2.2, 95 % CI 1.7–3.0), chronic bronchitis (OR = 1.5, 95 % CI 1.3–1.8), and emphysema (OR = 2.0, 95 % CI 1.7–2.4) [65]. In a large, population-based case–control study in women in Detroit, non-small cell lung cancer cases with a joint chronic obstructive lung disease phenotype were more likely to be white, heavy smokers, be exposed to environmental tobacco smoke, have childhood asthma, and have a history of asbestos exposure than lung cancer cases without a history of COPD [64]. Most epidemiologic studies of COPD, however, rely on self-report of COPD phenotype and are subject to both recall bias and misclassification.

Prospective studies have evaluated the association between computed tomography (CT) evidence of emphysema and/or spirometry-defined measures of airflow obstruction and risk of lung cancer, reducing the potential for disease misclassification. These studies report a 2- to 4-fold increased risk of lung cancer in the presence of CT evidence of emphysema, with no or lower risks associated with airflow obstruction [64, 73–75]. In studies using quantitative image analysis of CTs, no increased lung cancer risk among patients with emphysema was reported [76, 77]. Risk of lung cancer has also been shown to increase with decreasing forced expiratory volume in 1 second (FEV_1) even in smokers with only minimal declines in FEV_1 [72]. For these studies to move forward, consistently defined COPD will need to be evaluated in individuals with the joint COPD-lung cancer phenotype.

The lung cancer-COPD connection also is evidenced in family and genetic studies. First degree relatives of lung cancer patients show impaired FEV_1 [78] and a family history of COPD increases risk of lung cancer development [79], suggesting a common underlying genetic contribution to these diseases. In family-based genetic studies

for COPD, a region on 6q, just beyond the lung cancer linkage region and extending to the end of the chromosome, was linked to FEV_1 [80, 81]. There was also evidence for linkage of lung function to moderate obstructive lung disease in smokers on chromosome 12p [82, 83]. These data provide some regions of potential overlap in areas linked to lung function, COPD and lung cancer on chromosomes 6q and 12p.

Candidate gene studies in COPD and lung cancer have focused on inflammation, extracellular matrix proteolysis, and oxidative stress pathways [84–86], with some consistent findings for SNPs in epoxide hydrolase 1 (EPHX1), matrix metalloproteinases, and interleukin 1β (IL1B) [87–91]. Inflammatory pathway genes have been targeted for study because of the chronic inflammation caused by cigarette smoke. Van Dyke et al. showed that SNPs in *IL7R*, *IL15*, *TNF*, *TNFRSF10A, IL1RN*, and *IL1A* were associated with lung cancer risk in women with self-reported COPD, but not among women without COPD [92]. SNPs in *IL1A* have also been reported to be more strongly associated with lung cancer risk in those with emphysema [89]. GWAS for COPD-related phenotypes have identified some of the same regions identified in studies of lung cancer, namely 15q25.1 [50, 93–96]. Few studies, however, have evaluated a joint lung cancer and COPD phenotype. Young et al. summarize findings and report that the 15q25 locus is associated with risk of both diseases, genetic variation on 4q31 and 4q22 are associated with reduced risk of both diseases, loci on 6p21 are most strongly associated with lung cancer risk in smokers with COPD, and variants on 5p15 and 1q23 alter lung cancer risk when COPD is not present [97]. Taken together, these findings suggest that lung cancer occurrence is linked to COPD and more detailed studies of the joint phenotype using clearly defined COPD traits are needed to better untangle the relationship [98].

Infectious Agents

The role of infectious agents in lung cancer risk has had a varied focus over time with the changes in prevalent exposures. An association between TB and lung cancer has been reported for many years. In a meta-analysis of 37 case–control studies and 4 cohort studies, Liang et al. report significant associations between TB and subsequent lung cancer diagnoses, with risk estimates of 1.7 (95 % CI 1.5–2.0) adjusting for smoking history [99]. Similar risk estimates were reported in never smokers and highest risk was seen within 5 years of the TB diagnosis. In a more recent, population-based cohort study in Taiwan, a 1.8-fold increased risk of lung cancer was reported after a diagnosis of TB [100]. Human papillomavirus (HPV) infections have also been studied with regard to lung cancer risk. The prevalence of HPV in lung tumor tissue ranges from 0 % to 100 %, with great heterogeneity of findings across geographic regions, histology type of the lung cancer, sex, and HPV type [101, 102]. This is an area which will require additional study in high risk populations.

Another focus of current research is on lung cancer risk among individuals with HIV infection. With more effective treatments, HIV-infected patients are living

longer and lung cancer now ranks as one of the most frequently diagnosed non-AIDS-defining malignancies. Studies comparing lung cancer incidence in HIV-infected individuals to the general population have shown a 1.5 to 5.0-fold increased risk in infected individuals. In the review by Hou et al., standardized incidence ratios (SIRs) and incidence rate ratios (IRRs) adjusted for age, gender, race/ethnicity, smoking and route of infection are presented for 65 publications [103]. Lung cancer risk in HIV-positive populations varied with geographic region; SIRs or IRRs were 1.5–3.4 in Europe, 0.7–6.9 in the United States and 5.0 in Africa. Risk estimates were 5.4 in Europe and 2.8–3.0 in the United States for individuals with AIDS. Most studies showed little difference in lung cancer risk among HIV-infected patients receiving highly active antiretroviral therapy (HAART) and those not [104–106]. There are a number of limitations to the current body of literature, and continued follow-up of HIV-positive individuals will be needed to fully evaluate lung cancer risk that considers smoking more fully and focuses on race/ethnicity in populations at high risk of infection.

Risk Models

With the anticipated launch of population-based lung cancer screening on the horizon, identification of the group who would most benefit from early detection (i.e., those at highest risk of lung cancer) is critical to the success of a screening program. Over the last decade, various models have been proposed, as shown in Table 1. The eight proposed models are from both cohort (n = 4) [107–110] and case–control studies (n = 4) [111–114]. The majority of these studies are for current or former (usually defined as quit within one year of diagnosis or study entry) smokers, and use a combination of demographic characteristics (e.g., age and sex), intensity and/or duration of cigarette smoking, medical history, and occupational exposures as variables in the predictive model.

Discrimination was assessed by study authors using either concordance indices (c-statistic) or receiver operating curves (ROC), reported as area under the curve (AUC). Both provide an overall indication of the diagnostic accuracy of the model, with values closer to 1 indicating the model reliably distinguishes lung cancer cases from controls, whereas values at .50 indicate the predictor is no better than chance. It should be noted that while cross-validation (and the resulting concordance index) is useful to estimate the prediction capabilities of the proposed model, validation with an independent data set is preferred [115]. In addition, models presented discrimination measures for various time points (range, 1–10 years), so interpreting these values across models should be done with caution. Lastly, differences in the distribution of predictor variables included in the models that occur over time can affect model performance, as can cohort composition, case–control ascertainment and matching [116]. Thus, there is not one risk prediction model currently recommended for general use.

Table 1 Risk models for lung cancer in the general population

First author, (year)	Population type	Population details	Variables	Model discrimination	Other information
Bach et al. [107]	N = 18,172 Carotene and Retinol Efficacy Trial participants	All participants were either 20 pack year smokers (current or quit within 6 years) or asbestos-exposed men (current smokers or quit within 15 years)	-Age -Sex -Asbestos exposure -Duration of smoking -Cigarettes per day -Time since smoking cessation	One year risk: c-statistic = 0.72 Also presented a 10-year risk table	Within study validation and the Mayo Clinic CT study
Spitz et al. [112]	1851 lung cancer patients and 2001 control subjects (matched on age (±5 years), sex, ethnicity, and smoking status (never, former, or current))	Cases recruited from the Thoracic Center at The University of Texas M. D. Anderson Cancer Center Controls recruited from Kelsey – Seybold Clinics in metropolitan Houston, TX	*Never smokers*: -ETS -Family history of any cancer *Former smokers*: -Emphysema -Dust exposure -Hay fever -Family history of any cancer -Age stopped smoking *Current smokers*: -Emphysema -Pack-years -Dust exposure -Hay fever -Asbestos exposure -Family history of smoking-related cancer	One year risk : c-statistic: -Never smokers: 0.59 -Former smokers: 0.63 -Current smokers: 0.65	25 % of population used for validation
Spitz et al. [114]	725 lung cancer patients and 615 control subjects, (matched on age (±5 years), sex, ethnicity, and smoking status (current or former smokers only))	Cases recruited from the Thoracic Center at The University of Texas M. D. Anderson Cancer Center Controls recruited from Kelsey – Seybold Clinics in metropolitan Houston, TX	In addition to the variables listed above in Spitz (2007), data from two assays were included: a DNA repair capacity measure and a bleomycin sensitivity measure	Former smokers: AUC = 0.70 Current smokers: AUC = 0.73	Within study 3-fold cross-validation

Etzel et al. [113]	491 African American lung cancer patients and 497 African American control subjects (matched on age ±5 years), sex, ethnicity, and smoking status	Cases recruited from the Thoracic Center at The University of Texas M.D. Anderson Cancer Center Controls recruited from Kelsey – Seybold Clinics in metropolitan Houston, TX	-Smoking status -Pack years of smoking -Age at smoking cessation -Number of years since smoking cessation -COPD -Hay fever -Asbestos exposure -Wood dust exposure	AUC = 0.75 Five-year absolute risks also presented	Internal validation: 89 African American cases and 67 African American controls AUC = 0.75 External validation: Case–control studies of lung cancer in African Americans (172 cases and 153 controls) from metropolitan Detroit, Michigan AUC = 0.63
Cassidy et al. [111]	579 lung cancer cases and 1157 age- and sex-matched population-based controls	Residents of the Liverpool area	-Smoking duration -Pneumonia -Occupational asbestos exposure -Prior cancer -Family history of lung cancer	Five year risk: AUC = 0.71	No validation
Tammemagi et al. [109]	70,962 subjects in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO) and a subcohort of ever-smokers (N = 38,254)	Ten-site randomized screening trial in the US, enrollment started in November 1993 and ended June 2001. Subjects at study entry were men and women aged 55–74 years	-Age -Education -Body mass index -Family history of lung cancer -Chronic obstructive pulmonary disease -Recent chest x-ray -Smoking status -Pack-years smoked -Smoking duration -Smoking quit years (in ever-smoker subcohort)	Median follow-up = 9.2 years AUC = 0.86 (all) AUC = 0.81 (ever smoker)	Validation was performed with 44,223 PLCO intervention arm participants

(continued)

Table 1 (continued)

First author, (year)	Population type	Population details	Variables	Model discrimination	Other information
Hoggart et al. [108]	82,776 current smokers, 86,259 former smokers, 230,358 never smokers from the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort	Healthy participants from Italy, Spain, England, the Netherlands, Greece, Germany, and Norway between 40 and 65 years of age at study entry between 1993 and 1999	-Age -Average smoking intensity -Age started smoking -Duration of smoking (former smokers)	One year risk for smokers: Current: AUC = 0.82 Former: AUC = 0.83 Ever: AUC = 0.84 5 year risk: Current: AUC = 0.77 Former: AUC = 0.72 Ever: AUC = 0.79	Data split into test (10 %) and training (90 %) sets Examined various nonsmoking exposures but did not include in final model
Tammemagi et al. [110]	73,618 ever-smoking subjects in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial	Ten-site randomized screening trial in the US, enrollment started in November 1993 and ended June 2001. Subjects at study entry were men and women aged 55–74 years	-Updated to exclude chest X-ray and include race and prior cancer	Six-year risk: AUC = 0.80 (36,286 ever smokers)	Internal validation: 37,332 PLCO smokers AUC = 0.80 External validation: 51,033 National Lung Screen Trial participants (all ever smokers) AUC = 0.70

In addition to these published reports using risk factors based on demographic and exposure information, limited research has been published incorporating genetic information (e.g. SNPs) into risk prediction models. Overall, the addition of SNPs into currently available models does not significantly improve model performance [117–119]. Given that lung cancer is a complex, polygenic disease with a strong environmental component associated with risk, these findings are not unexpected. Theoretical studies in other types of cancer suggest that common SNPs are unlikely to be clinically useful to include in building models to help inform risk-based interventions, such as screening [120].

Summary

Cigarette smoking continues to be the overwhelming risk factor for lung cancer. While lung cancer incidence and mortality rates have declined with a decrease in cigarette use, lung cancer remains a disease that is diagnosed at later stages with very poor survival. Several risk factors, other than smoking, have been identified and include environmental exposures such as radon and asbestos, as well as, family history and genetic susceptibility. Further understanding of the roles of a previous diagnosis of COPD, marijuana, hookah and e-cigarette use, and infections are needed to better define the highest risk group. The advent of screening for lung cancer using low dose CT will result in decreased mortality and will be most cost-effective in a well-defined high risk population.

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Lung Cancer in Never Smokers

Gabriel Alberto Rivera and Heather Wakelee

Abstract Lung cancer is predominantly associated with cigarette smoking; however, a substantial minority of patients with the disease have never smoked. In the US it is estimated there are 17,000–26,000 annual deaths from lung cancer in never smokers, which as a separate entity would be the seventh leading cause of cancer mortality. Controversy surrounds the question of whether or not the incidence of lung cancer in never-smokers is increasing, with more data to support this observation in Asia. There are several factors associated with an increased risk of developing lung cancer in never smokers including second hand smoke, indoor air pollution, occupational exposures, and genetic susceptibility among others. Adenocarcinoma is the most common histology of lung cancer in never smokers and in comparison to lung cancer in smokers appears less complex with a higher likelihood to have targetable driver mutations.

Keywords Non-smoker lung cancer • Lung cancer in nonsmokers

Introduction

Lung cancer is strongly associated with cigarette smoking [1–3]; however, there is a substantial minority of patients who have never smoked. This population is more likely to have distinct molecular markers [4–7], but has less well established risk factors adding to the complexity in understanding this subset. The epidemiology, risk factors, molecular biology, treatment and prognosis of lung cancer will be discussed in never-smokers. Small cell lung cancer in never-smokers is incredibly rare with only case reports and series published; therefore, we will focus on non-small cell lung cancer (NSCLC) [8–11]. In addition, from this point forward a person who is a never smoker is identified as having smoked less than 100 cigarettes in a lifetime.

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Epidemiology-Defining the Never Smoker Population

Lung cancer prior to the invention of mechanized cigarette making was a rare disease. What percentage of patients with the disease were never-smokers is unclear; however, inhaled tobacco was available on a limited basis [12, 13]. The capacity to identify never-smokers in numbers meaningful for global and regional analysis to date has been limited as several cancer registries have inconsistently identified smoking status. The studies that exist with data on never-smokers have often had to resort to creative ways of estimating smoking prevalence.

What Is the Incidence and Mortality of Lung Cancer in Never-Smokers?

In 2012, there were an estimated 1.8 million new cases of lung cancer reported by GLOBOCAN [14]. In actuality the incidence varies dramatically by continent or region [15–18]. Given the high fatality rate of lung cancer, mortality closely tracks incidence with 1.59 million deaths projected globally for 2012 [14]. In the US the incidence of new lung cancer cases for 2014 is estimated at 224,210 and deaths estimated at 159,260 [19]. Reports in the US have estimated among never-smokers annual deaths of 17,000–26,000 from lung cancer that as a separate entity would be the seventh leading cause of cancer mortality [7, 20].

Is Incidence or Death Rate of Lung Cancer in Never Smokers Increasing? Is There a Difference by Sex or Ethnicity?

There have been very few studies that have been able to accurately report incidence rates of lung cancer in never-smokers, in particular across regions. Sun et al. and Subramanian et al. report that in the year 2000 never-smokers were 25 % of all cases of lung cancers globally, of which 15 % were men and 53 % were women [6, 7]. These percentages, however, are actually the inverse of percentages taken from the Parkin et al. study of smoking related lung cancer [21]. In contrast, Wakelee et al. performed direct measurement of the incidence of lung cancer in never-smokers by utilizing data from six large cohorts primarily from the United States and Sweden from 1971 to 2002. Age-adjusted incidence rates by sex for people aged 40–79, ranged from 4.8 cases for men in the Swedish cohort to 20.8 per 100,000 person years for women in the California Teachers Study cohort; however, the study was not designed to answer whether the incidence rates were increasing over time [22]. The incidence rates were higher for women than they were for men, but this does not answer whether women who never smoke are at an increased risk to develop lung cancer compared to men [23]. Thun et al. tried to answer this question of whether the incidence rate of lung cancer was increasing by comparing historical

data to more recent analyses. Taking data from 13 large cohorts and 22 cancer registries they examined cohorts with low prevalence of smoking either reported from 1983–1987 or historical cases of US women in the 1935–1940 Connecticut Tumor Registry. It was noted in women of European descent the age-standardized rate for lung cancer in never-smokers was 9.7 per 100,000 women, which was similar to Basque women at 8.6 in the 1980s and 8.7 in US women in the 1930s [18]. Although this study demonstrated no increase in the incidence of lung cancer in never-smoker women over time, it had limited numbers to report incidence rates accurately for Asians, and African Americans, and made no comment on men. A single center study in Asia reported a significantly increased incidence of lung cancer in never-smokers spanning three decades from 1970 to 2000 that went from 15.9 % to 32.8 % over this time period [24]. Caution should be taken; however, as this study reported only proportions, not incidence rates or standardized rates. Unfortunately, to date lung cancer incidence in never-smokers has yet to be studied in an accurate and comprehensive manner given the lack of smoking data in the majority of cancer registries.

Several large studies have examined death rates from lung cancer in never-smokers stratified by sex and ethnicity, specifically examining temporal trends as well as risk. Death rates by sex were examined in the two large American Cancer Society (ACS) studies, Cancer Prevention Study (CPS) I (1959–1972) and II (1982–2000), to give age-specific and age-standardized death rates for 460,000 never-smokers, aged 35–84, all within the US, Puerto Rico, and Guam. They found age-standardized death rates were significantly higher in men at all ages in the first CPS cohort. In the second cohort the higher death rate in men was only seen in those age 60 and above, which may have been related to the decreasing death rate among men, ages 35–69, and the increased death rate in women, ages 70–84 [20]. The study mentioned previously by Thun et al. included CPS I and extended the coverage of CPS II by 4 years and reported higher death rates in men aged 40 and over in those of European and Asian descent. The number of deaths in African American men was small, making it difficult to reliably comment on all age groups. In never-smokers of European descent in the US, the calculated risk to develop lung cancer was estimated at 1.1 % for a man and 0.8 % for a woman before the age of 85 [18].

Limited data exists on comparative analysis between ethnic groups among never-smokers with lung cancer. In the ACS cohort analysis, incidence was only significantly higher for African American women aged 40–69 compared to men and women of European descent [18]. Gomez et al., in a population-based case-control study from Northern California, found that from the years 1998–2003 and 2005–2008, a higher proportion of female never smokers with lung cancer were Asian Pacific Islanders (API) and Latinas compared to non-Hispanic whites. Although this was a small study, hazard ratios were calculated and a cox proportional hazard model demonstrated that mortality rates in U.S. born Latinas and female API were 2.1 and 1.7 times higher, respectively, than non-Hispanic whites [25]. Caution in interpretation must be taken given significant variance between regions. This was illustrated by Liu et al. who found significant variation in the incidence of lung cancer in never-smokers in China, even in neighboring cities. Taking all the cities together the death rate was 0.5 per 1000 never-smokers compared to 1.5 in smokers [17].

Potential Risk Factors in the Development of Lung Cancer in Never Smokers

Second Hand Smoke

The IARC as well as several authors list second hand smoke (SHS) as carcinogenic with an excess of lung cancer from SHS as high as 25 % [26–28] (Table 1). A descriptive study found that amongst those with SHS exposure approximately 22 % were men and 88 % were women [29]. While frequency data does not evaluate association or risk there have been several case-control studies that have calculated

Table 1 Selected risk factors in the development of lung cancer in never smokers

Risk factor	Risk ratio	Comments	References
Second hand smoke	1.34	Prospective nested case control in Europe	[27]
	1.23 (spouse) 1.27 (workplace)	Pooled analysis from two case controls US & Europe	[31]
	2.1	Case control from Toronto	[32]
	1.34	Prospective cohort study in Japan	[34]
Residential radon	0.18 per 100 Bq/m3	This is an estimated odds ratio based on unit of radioactivity exposure from radon measured in Becquerel (Bq)	[43]
Indoor air pollution (biofuel)	2.15	Meta-analysis of 25 case control studies of household coal	[54]
Occupational exposures	3.49 asbestos 2.48 paint dust	Prospective cohort study in the Netherlands	[58]
Lung disease	2.93	Asthma	[57]
Genetic susceptibility	1.95	Family history of lung cancer in first degree relative	[65]
	1.62-Heterozygote 2.35-Homozygote	5p15.33 Asian females	[73]
	5p15.33: White-1.15 Asian-1.23	Pooled analysis of 21 case control studies of Whites and Asians. 15q25.1 & 6p21.33 were not significantly associated with risk of lung cancer in never smokers.	[77]
	0.68	18p11.22 447 Korean never smokers	[79]
	1.46	13q31.3 multicenter study in US	[78]
	1.32 0.85 1.16 1.19 0.86	10q25.2 6q22.2 6p21.32 3q28 17q24.3 Study population were Asian women	[80]
	0.45	MSH2	[89]

MSH2 mutS homolog 2

odds ratios and utilized unconditional regression analysis to help answer this question. Unfortunately, not every study lists this information in the analysis; therefore, caution should be taken for over estimation of odds ratios [30]. In these case-control studies SHS for the most part has been subdivided into home, workplace, or social with differences noted in odds ratios from one setting to the other, but all with odds ratios greater than 1 and higher risk noted with combined exposures noted in regression analysis [31–34]. As SHS is often assessed by interview from smoking partner, non-smoking partner, or next-of-kin the validity of these measures as well as non-smoker status have been brought into question; however, misclassification is likely low [35, 36]. Other studies have been able to demonstrate dose response by SHS intensity and duration [31, 34]. One author also noted an increased risk if exposed to SHS prior to the age of 25 [37].

Indoor Pollution

Indoor air pollution includes: radon from soil and water, products of combustion such as coal, chemicals from household products, and biological agents such as mold among many other sources [38]. This is a fairly large category of which the focus in this section will be on those that are known to cause an increased risk of lung cancer.

Radon is a decay product from radium that is found in rock and soil. Radon can further decay into polonium that enters the air and water and emits alpha particles, which can cause DNA damage [39]. While it has already been established that in miners radon exposure can lead to lung cancer [40], less well defined is whether low-indoor radon levels are associated with increased risk as well. The following presentation of studies will examine different methods of measuring association and risk with varying results to answer this important question.

Krewski et al. in a combined analysis of seven large case-control studies in North America, including 4081 cases and 5281 controls, demonstrated with estimated odds ratios (EOR) that residential exposure to radon in general was associated with the lung cancer risk. It should be noted that although the odds ratios were numerically positive and statistically significant in two out of the seven studies, in the pooled analysis there was no statistically significant difference [40]. Arguably, relative risk is a better measure to assess causation than excess risk of disease as had been used in the study above [41]; however, both assess association and to assess causality is more complex requiring the achievement of specific criteria [3]. Sandler et al. was not able to find statistically significant excess relative risk of lung cancer related to radon exposure at any level [42]. This study, however, had significantly lower radon levels on average that did not even meet the actionable level suggested by the EPA. This study was also underpowered to assess whether there was any synergistic effect in smokers. In contrast, two separate studies from Darby et al. and Leuruad et al. found the relative risk of lung cancer in never smokers increased with radon exposure [43, 44]. One study identified a potentially high-risk group as those

who were homozygous for glutathione-S-transferase M1, an enzyme responsible in neutralizing reactive oxygen species [45].

Several studies have established a large variation in the incidence of lung cancer in Asian countries that has been postulated to be possibly due to unreported tobacco use, but also likely due to indoor air pollution [17, 18]. Indoor air pollution has been examined as a global health issue associated with an increased risk of lung cancer along with other respiratory illnesses [46]. The risk for lung cancer varies, but has been found largely in developing countries [6, 7]. Coal and wood smoke are now recognized by the IARC as a human carcinogen [47]. The use of indoor combustion products is highest in Africa and South East Asia at greater than 60 % compared to the Americas and Europe at less than 20 % [48]. Kleinerman et al. interviewed men and women from two prefectural areas in Northwest China in a case-control study on the use of coal and biomass fuel in heating and cooking in controls and lung cancer patients. They adjusted for smoking status and frequency matched for age and sex, and find a modest increased risk for those with the highest exposures [49]. In a retrospective analysis from the Yunnan Province of China, the authors found a significantly increased absolute and relative risk of dying from lung cancer in those who utilized smoky coal versus smokeless coal [50]. Coal use was also evaluated in a large case-control study in participants from Eastern/Central Europe and the United Kingdom where there was an increased risk observed when solid biofuels (coal or wood) were used for cooking [38]. This was confirmed in a large meta-analysis performed of 25 case control studies that covered cases from Africa, North America, Europe, India, Mainland China and Taiwan. Although there were differences in the risk of lung cancer by regions, the overall trend was an increase in lung cancer risk in particular in parts of China and Taiwan [51]. A transition to ventilated stoves was associated with a decreased in lung cancer incidence in at least one analysis from China [52].

Occupational Exposures

Several authors have examined occupational exposures such as pesticides, grain elevator dust, wood dust, smoke soot or exhaust as risk factors for lung cancer in never-smokers [32, 53, 54]. A large prospective cohort study of men from The Netherlands assessed cumulative probability of exposure to four specific known carcinogens at the work place reported by the IARC: asbestos, paint dust, polycyclic aromatic hydrocarbon (PAH), and welding fumes [54]. After adjusted for age and smoking status in the final analysis they found a significantly increased risk if exposed to asbestos or paint dust, with asbestos having the highest risk. They also found that the tested population was fairly representative of the Dutch population and that 11.6 % and 1.7 % of the lung cancers were attributable to asbestos and paint dust, respectively [54]. None-the-less, occupational exposure does not explain fully lung cancer in never-smoker as it has been shown that individuals without any occupational or environmental exposure can develop lung cancer [12, 29].

Lung Disease

Epidemiologists have studied whether specific lung conditions or infections are associated with an increased risk of lung cancer, but with conflicting data likely due to the confounding factor of smoking status. One study found no increased risk for lung cancer in never-smokers who had emphysema, chronic bronchitis, asthma, pneumonia, or tuberculosis [32]. Another study found asthma in never-smokers was associated with increased risk of lung cancer with an odds ratio of 2.93 compared to those without asthma [53]. An increased risk has also been seen in those with a history of tuberculosis infection with an odds ratio as high as 3.5 in never-smokers, particularly for disease on the same side of the previous infection [55, 56]. The association in individuals with pulmonary fibrosis is also unclear [57–60].

Radiation Exposure

In patients exposed to ionizing radiation either as treatment for breast cancer or Hodgkin's disease there is an increase risk in the development of lung cancer [61–63]. A recent study reports that molecular rearrangement of the *RET* gene may explain a small percentage of radiation induced adenocarcinomas of the lung in never-smokers [64].

Genetic Factors

Environmental exposures as described above appear to increase the risk of lung cancer in never-smokers and genetic factors play as role as well. Nitadori et al. performed a large prospective cohort study of a Japanese population to examine whether family history in a first degree relative increased the risk of lung cancer. A family history of lung cancer was associated with a significant increase in risk of lung cancer in both ever and never smoker groups, although the risk was higher in women and in never-smokers [65]. It should be noted this study controlled for SHS, but did not compare hazard ratios between ever-smokers versus never-smokers to examine whether there was a significant difference between groups. Several other studies have also reported similar results in cohort or case-control analyses [32, 33, 53, 66, 67].

On the molecular level, Bell et al. noted a family of European descent with a germline *epidermal growth factor receptor* (*EGFR*) mutation. The proband as well as his brothers, had a T790M *EGFR* germline mutation, but the two brothers did not have lung cancer. The proband at surgery was noted to have five separate tumors that were analyzed for somatic mutations in the *EGFR* domain with the missense L858R mutation and in-frame deletion delL747-T751 noted [68]. Ohtsuka et al.

identified a separate germ-line mutation in *EGFR*, V843I, which was found in multiple generations as an identical mutation. As in the previous study, in addition to the germline mutation, somatic mutations were identified including L858R. Interestingly, the V8438I mutation confers resistance to tyrosine kinase inhibitors as has been described for the T790M mutation; however, the exact mechanism for this resistance or increased susceptibility to lung cancer from either mutation has yet to be elucidated [69]. Recently, a unique *HER2* germline mutation, G660D, was identified in a Japanese family with lung cancer in multiple generations [70].

While these last two studies have examined the potential heritability of lung cancer risk at a specific gene level, other studies have used more traditional linkage analysis strategies to identify loci of interest that might confer risk to the disease [71]. One study utilized comparative genomic hybridization analysis to determine that in never-smokers of Chinese descent with lung cancer, gain of 16p was frequent, though loss of 16p was identified in an earlier study that was not restricted to patients of Chinese ancestry [71, 72]. Several scientists have performed genome wide association studies (GWAS) in never-smokers utilizing single nucleotide polymorphism array data and have identified 5p15.33 locus (*TERT-CLPTM1L*) as one that confers an increased risk of lung cancer [73–75]. There have been several other loci identified; however, they have not been consistently replicated, perhaps due to ethnic or environmental exposure differences [76, 77]. This was seen in two separate studies where 13q31.3 (*GPC5*) was identified in an American population [78] and 18p11.22 (*FAM38B*) in a Korean population [79]. A large GWAS among Asian females found several novel chromosomal aberrations at 10q25.2 (*VTI1A*), 6q22.2 (*ROS1 & DCBLD1*), and 6p21.32 (*HLA-DRA*) when compared to controls [80]. They also confirmed two other mutations, 3q28 (*TP63*) and 17q24.3 (*BPTF*), reported in three separate studies [81–83]. Other scientists have focused on specific genes that have largely been associated with the metabolism of tobacco related carcinogens in the cytochrome P450 system. The data has been inconsistent with some [84, 85] reporting an increased risk in those with CYP1A mutations while others have refuted this finding [86]. Other studies have looked at DNA repair with some data to support an increased risk in those with the lowest DNA repair capacity [87], while others have implicated polymorphisms in the ataxia telangiectasia mutated gene [88] or mismatch repair gene MSH2, in particular if associated with SHS exposure [89, 90]. Govindan et al. performed whole genome sequencing as well as whole transcriptome sequencing, on multiple lung cancer surgical specimens, including in some tumors from never smoking patients. This study was able to demonstrate significant difference in mutation rate between smokers and never smokers indicating a different oncogenic process [91].

Pathology

The topic of histological subtype has been well studied with the majority of data reporting adenocarcinoma as the most common histological type in never smokers [6, 7, 22, 92, 93]. In a review paper on lung cancer in never-smokers

Samet et al. presents relevant theories of why a trend for an increase in adenocarcinoma has been observed, even in smokers, and especially in women, including change in puff volume that may distribute carcinogens differently within lung tissue as well as increased nitrate levels due to greater combustion of tobacco material within the cigarette, which he asserts also impacts never-smokers through side stream smoke [12]. One study looked at subtypes of adenocarcinoma as they correlate with common mutational status among never-smokers [94].

Driver Mutations

Considerable research has led to identification of "driver mutations" in adenocarcinoma of the lung and two, *EGFR* and *ALK*, already have FDA approved therapeutics. Others such as *ROS1*, *BRAF* and *HER2* are targetable with drugs on the market for other indications and more, including *KRAS* and *RET*, with agents that are in ongoing investigations. Specifics on molecular profiling and receptor cell signaling will be discussed in detail in chapters later in this book.

Estimates of actionable mutational frequency in never-smokers vary by ethnicity or region (Fig. 1) with reports as high as 90 % in a single-center institution of Chinese women with lung cancer that included analysis for *EGFR*, *ALK*, *HER2*, and *KRAS* [94] to 29.4 % EGFR mutant in a recent evaluation of 907 patients with lung cancer from India [95]. In general, mutations within *EGFR* are identified in approx-

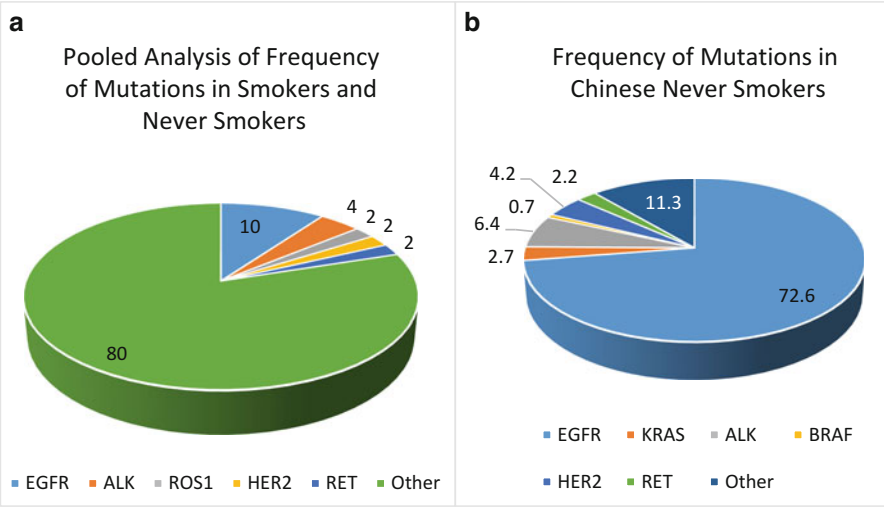

Fig. 1 Mutations are more common in never smokers with adenocarcinoma. In figure (**a**), percentages are displayed of the frequency of mutations in adenocarcinoma that includes smokers with never smokers. Figure (**b**), although inherent biases exist in single center studies, this single center study in China illustrates the proportion of identifiable mutations in a population of 408 never smokers with adenocarcinoma

imately 10 % of lung adenocarcinomas, but at a much higher frequency in never-smokers [96]. Another example is that HER2 exon 20 insertion mutations constitute approximately 2 % of NSCLC adenocarcinoma mutations but are more common in women, with adenocarcinoma who were never-smokers [97, 98].

Other significant mutations found in lung cancer, with a higher frequency in those who develop lung cancer as never-smokers, are ALK and ROS1 [99–101]. ALK mutations represent approximately 4 % of lung adenocarcinomas while ROS1 is around 2 %. Patients with this mutation also appear to be younger, never smokers, and have adenocarcinoma [97]. RET alterations are found in roughly 2 % of adenocarcinomas of the lung with individuals typically younger and never-smokers [64, 102, 103].

Prognosis

Survival data for lung cancer patients who are never-smokers as compared to smokers is conflicting. Subramananian et al. in a single-center case-control study did not find a survival difference between smokers and never smokers [104]. Nordquist et al. in a single center study however, reported 16 % vs 23 % 5-year survival rates in smokers compared to never-smokers. Smoking was a negative predictive value on regression analysis in another study [105]. A very large retrospective study by Kawaguchi et al. utilizing 15,185 Japanese individuals from one national registry and 13,332 Caucasians from a cancer registry in Southern California found a statistically significant survival advantage for Japanese never-smokers and a trend for Caucasian never-smokers compared to smokers with lung cancer [106].

Summary

Lung cancer in never smokers as a separate entity is the seventh leading cause of cancer related mortality. There appears to be racial variation in the incidence and mortality that require further research. Established risk factors include second hand smoke, several environmental toxins and potential genetic predispositions, but much work still needs to be done in this area. Whatever the inciting event, it appears adenocarcinoma is the most common histological type and can be associated with a variety of somatic mutations with important therapeutic implications.

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Immune Therapy

Lysanne Lievense, Joachim Aerts, and Joost Hegmans

Abstract Lung cancer has long been considered an unsuitable target for immunotherapy due to its proposed immunoresistant properties. However, recent evidence has shown that anti-tumor immune responses can occur in lung cancer patients, paving the way for lung cancer as a novel target for immunotherapy. In order to take full advantage of the potential of immunotherapy, research is focusing on the presence and function of various immunological cell types in the tumor microenvironment. Immune cells which facilitate or inhibit antitumor responses have been identified and their prognostic value in lung cancer has been established. Knowledge regarding these pro- and anti-tumor immune cells and their mechanisms of action has facilitated the identification of numerous potential immunotherapeutic strategies and opportunities for intervention. A plethora of immunotherapeutic approaches is currently being developed and studied in lung cancer patients and phase 3 clinical trials are ongoing. Many different immunotherapies have shown promising clinical effects in patients with limited and advanced stage lung cancer, however, future years will have to tell whether immunotherapy will earn its place in the standard treatment of lung cancer.

Keywords Cancer immunotherapy • Tumor microenvironment • Immunosuppressive cells • Regulatory T cells • Myeloid-derived suppressor cells • Tumor-associated macrophages • Tumor antigens • Tumor vaccines • Cellular immunotherapy • Personalized medicine

Introduction

Cancer immunotherapy consists of approaches that enhance the host immune system to generate effective immune responses against cancer. The editors of Science have chosen this strategy to combat tumors as the Breakthrough of the Year for 2013 [1]. Although it is still in its infancy, over the last decade we have witnessed that

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immunotherapeutic approaches are becoming more appealing components of the anticancer armamentarium. In 2010, sipuleucel-T became the first therapeutic vaccine to be approved by the US FDA for the treatment of metastatic, hormone-refractory prostate cancer [2]. This was followed in 2011 by ipilimumab, a fully human monoclonal antibody which blocks cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), the first agent approved in the EU for the treatment of unresectable or metastatic melanoma [3]. Examples of immune modulation in lung cancer are the MAGE-A3, L-BLP25 and TG4010 vaccines and belagenpumatucel-L; these are discussed in detail below. With the increasing understanding of the fundamentals of cellular and molecular tumor immunology, many ways are now investigated how the immune system can be further augmented to treat lung cancer. However, to understand the principles of these novel immunotherapeutic approaches, it is important to comprehend the different immune cell components and their function in the tumorigenic process. Most of these cells will be discussed in the next section, where it will become clear that the immune system plays a dual role in carcinogenesis. The complex interactions between diverse immune cell types and tumor cells can actively favor tumor rejection as well as tumor progression, depending on the tumor cell characteristics, type, stage, secreted factors and the types of immune cells that are involved. It emphasizes the importance of the full understanding of the intricacy of the cellular interactions within the tumor microenvironment as well as the effects of tumor-derived factors on distant involved immunological tissues and organs. Understanding the local and systemic immune mechanisms will lead to new potential therapeutic targets. There is a rapid progress in this field of cancer immunology and the development of novel immunotherapeutic approaches. Therefore, immunotherapy is by many considered as the fourth modality in conventional cancer treatments alongside surgery, chemotherapy and radiation. It is believed that the role of immunotherapy for lung cancer treatment is in combination regiments with surgery (debulking of the tumor), chemotherapy (inducing immunogenic cell death), and merged immunotherapeutic approaches that further modulate the tumor-host equilibrium towards tumor regression [4, 5].

Immune Cell Involvement in Lung Cancer Carcinogenisis

There is currently overwhelming evidence that several immunological cell types of the host influence lung cancer incidence, cancer growth, response to therapy and thereby the prognosis of the disease. Immunohistochemical analysis of lung cancer biopsies have demonstrated that the tumor microenvironment is a heterogeneous and complex system of tumor cells and stromal cells, including endothelial cells and their precursors, pericytes, smooth-muscle cells, and fibroblasts of various phenotypes, located within the connective tissue or extra-cellular matrix (e.g. collagen). Leukocyte infiltration is an important characteristic of cancer and the main components of these infiltrates include natural killer (T) cells, mast cells, neutrophils, B- and T lymphocyte subsets, myeloid derived suppressor cells, macrophages and dendritic cells (Fig. 1). Based on their functions, these cells can be divided into cells

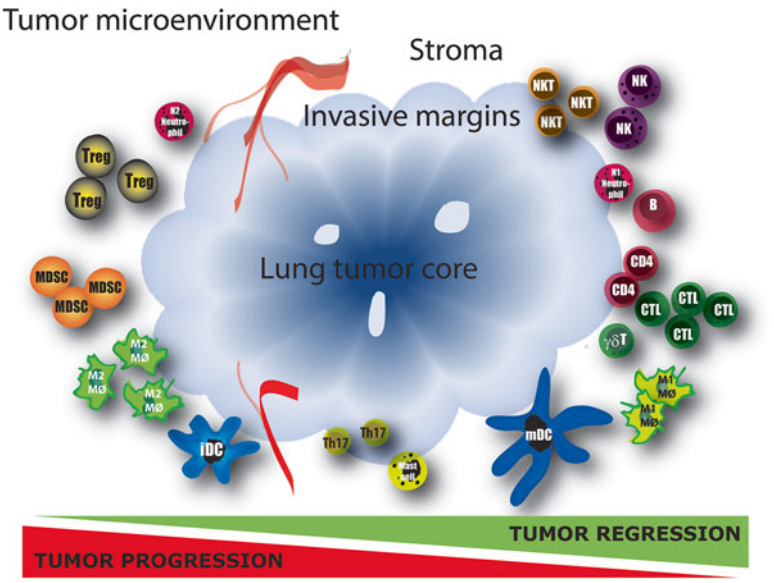

Fig. 1 The tumor microenvironment is a heterogeneous and complex system of tumor cells and 'normal' stromal cells, including endothelial cells and their precursors, pericytes, smooth-muscle cells, and fibroblasts of various phenotypes, located within the connective tissue or extra-cellular matrix (e.g. collagen). Leukocyte infiltration is an important characteristic of lung cancer and the main components of these infiltrates include natural killer (T) cells (NK/NKT), N1/N2 neutrophils, B- and T-lymphocyte (cytotoxic T cells [CTL]; T helper 17 [Th17]; CD4; regulatory T cells [Treg]; gamma delta T cells) subsets, myeloid derived suppressor cells (MDSC), M1/M2 macrophages (MØ), mast cells, and immature dendritic cells (iDC)/mature dendritic cells (mDC). Based on their functions, these cells can be divided into cells with a potentially positive impact on the antitumor response (right) and cells with a detrimental effect (left). The net effect of the interactions between these various cell types and their secreted products within the environment of an established tumor participates in determining anti-tumor immunity, angiogenesis, metastasis, overall cancer cell survival and proliferation

with a potentially positive impact on the antitumor response and cells with a detrimental effect. The net effect of the interactions between these various cell types and their secreted products within the environment of an established tumor participates in determining anti-tumor immunity, angiogenesis, metastasis, overall cancer cell survival and proliferation. Next we will provide a short update on the function of these immune cells for lung cancer that is adapted from Heuvers et al. [6].

Natural Killer (T) Cells

Natural killer (NK) cells (expressing the surface markers CD16 and CD56, but not CD3) are lymphocytes that play an important role in the rejection of tumors without previous sensitization and without restriction by the major histocompatibility

complex (MHC) [7, 8]. NK cells eradicate tumors through multiple killing pathways, including direct tumor cell killing. They also secrete cytokines and chemokines like interleukin (IL) IL-10, tumor necrosis factor (TNF)-α, and the principal NK-derived cytokine interferon (IFN)-γ, which can coordinate the innate and adaptive immune responses to tumor cells and may lead to apoptosis of the attacked cells. A large cohort study showed that an increase in NK cells in tumor tissue is a strong independent prognostic factor for the survival of lung cancer patients [9]. Natural killer T (NKT) cells (CD16+, CD56+, CD3+) are a subset of NK cells that are found in the peripheral blood, tumor tissue and pleural effusions of lung cancer patients in decreased numbers and with reduced functions [10, 11]. It has been shown that NKT cells in cancer patients produce a decreased amount of IFN-γ and are therefore less effective than NKT cells in healthy controls [12, 13].

Mast Cells

Accumulation of mast cells is common in angiogenesis-dependent conditions, like cancer, as mast cells are major producers of proangiogenic molecules as vascular endothelial growth factor (VEGF), IL-8, transforming growth factor (TGF)-β [14]. Mast cells also play a central role in the control of innate and adaptive immunity by interacting with B and T cells (in particular regulatory T cells) and dendritic cells. The density of mast cells in non-small cell lung cancer (NSCLC) tumors is correlated with microvessel density [15] and mast cells/histamine has a direct growth promoting effect on NSCLC cell lines in vitro [16]. Tumor-infiltrating mast cells can directly influence proliferation and invasion of tumors, by histamine, IL-8 and VEGF while the production of TNF-α and heparin can suppress tumor growth [16, 17]. It has been shown that in NSCLC mast cell counts were enhanced as tumor stage increased while another study did not found this correlation [14, 18]. The controversy of mast cells in other cancer types seems to be related to the type, microenvironment and stage of cancer and their role may depend on the tumor environment [19, 20].

Neutrophils

Neutrophils play a major role in cancer biology. They make up a significant portion of the infiltrating immune cells in the tumor and the absolute neutrophil count and the neutrophil to lymphocyte ratio in blood are independent prognostic factors for survival of NSCLC [21–23]. Neutrophils are attracted to the tumor under the influence of specific chemokines, cytokines and cell adhesion molecules. Tumor-associated neutrophils (TAN) have polarized functions and can be divided into the N1 and N2 phenotype in a context-dependent manner [24]. The N1 phenotype inhibits tumor growth by potentiating T cell responses while the N2 phenotype

promotes tumor growth [25]. The antitumor activities of N1 neutrophils include expression of immune activating cytokines (TNF-α, IL-12, GM-CSF, and VEGF), T cell attracting chemokines (CCL3, CXCL9, CXCL10), lower expression of arginase, and a better capacity of killing tumor cells in vitro. N2 neutrophils support tumor growth by producing angiogenic factors and matrix-degrading enzymes, support the acquisition of a metastatic phenotype, and suppress the anti-tumor immune response by inducible nitric oxide (NO) synthase and arginase expression [26]. Neutrophils also influence adaptive immunity by interacting with T cells [27], B cells [28], and DC [29]. In resectable NSCLC patients, intratumoral neutrophils were elevated in 50 % of the patients and this was associated with a high cumulative incidence of relapse [30].

B Lymphocytes

B cells may affect the prognosis of patients with lung cancer, as patients with stage I NSCLC contain more intratumoral germinal centers with B lymphocytes than patients with stages II to IV [31]. These tertiary (T-BALT) structures provide some evidence of an adaptive immune response that could limit tumor progression in some patients. For instance, the production of antibodies by B cells can activate tumor cell killing by NK cells and other inflammatory cells [32]. Auto-antibodies against tumor antigens are commonly found in patients with lung cancer [33–35] and can inhibit micrometastasis [36]. The role of B cells seems depending on the context.

T Lymphocytes

Several subsets of T cells have been identified, and they all develop in the thymus from common precursor T cells. Based on cytokine secretion and function, these cells are classified as CD4, CD8, Th17, regulatory T cells (Tregs), or TH17 cells, amongst others. CD4+ cells and CD8+ cells represent the strong effectors of the adaptive immune response against cancer [37]. There is controversy on the impact of T cells and their localization on the prognosis of lung cancer [38–43]. This may be caused by the presence of a special subset of T cells, the regulatory T cells (Tregs), and myeloid-derived suppressor cells which are both discussed below. Also tumor-derived factors can exhaust T lymphocytes or induce their apoptosis [44]. Recently it has been shown that cytotoxic T lymphocytes (CTL) within the tumor (the tumor-infiltrating lymphocytes [TIL]) are of beneficial prognostic influence in resected NSCLC patients in both adenocarcinoma [45] and squamous cell carcinoma [46].

Tregs, characterized by the expression of CD4, CD25, Foxp3, but absence of CD127, are T lymphocytes that are generated in the thymus (natural Treg) or induced

in the periphery (induced Treg) when triggered by suboptimal antigen stimulation or stimulation with IL-35, TGF-β and IL-10 [47]. Tregs are further characterized by the expression of glucocorticoid-induced TNF-receptor-related-protein (GITR), lymphocyte activation gene-3 (LAG-3), and cytotoxic T-lymphocyte-associated antigen 4 (CTLA4). In cancer patients, Tregs confer growth and metastatic advantages by inhibiting anti-tumor immunity. They have this pro-tumoral effect by promoting tolerance via direct suppressive functions on activated T-cells or via the secretion of immunosuppressive cytokines such as IL-10 and TGF-β [48, 49]. Tregs are present in tumor tissue [50, 51] and increased in peripheral blood of NSCLC patients compared to healthy controls [52, 53]. This increase in Tregs was found to promote tumor growth and was correlated with lymph node metastasis [54, 55] and poor prognosis [50, 56]. Many factors can increase Tregs in NSCLC tumors, among them are thymic stromal lymphopoietin (TSLP) [57] and intratumoral cyclooxygenase-2 (COX-2) expression [58]. Tregs are considered the most powerful inhibitors of antitumor immunity [59].

Th17 cells are a subpopulation of CD4+ T helper cells that are characterized by the production of interleukin-17 (IL-17, also known as IL-17A). IL-17 plays an important role in the host defenses against bacterial and fungal infections by the activation, recruitment, and migration of neutrophils [60, 61]. In vitro experiments have shown that IL-1β, IL-6, and IL-23 promote Th17 generation and differentiation from naïve CD4+ T cells [62]. Among the other cytokines secreted by Th17 cells are IL-17 F, IL-21, IL-22, and TNF-α. The role of Th17 cells in cancer is poorly understood. Th17 cells accumulate in malignant pleural effusion from patients with lung cancer [62]. Also higher levels of IL-17A were detected in serum and in tumor lesions of lung adenocarcinoma patients, indicating a potential role of these cells in cancer [63]. It has been shown that Th17 cells encouraged tumor growth by inducing tumor vascularization or enhancing inflammation, but other studies revealed also opposite roles for Th17 cells. Recent data indicate that IL-17 may play a role in the metastasis of lung cancer by promoting lymph-angiogenesis and is therefore an independent prognostic factor in both overall and disease-free survival in NSCLC [64]. So, it is controversial whether Th17 cells in cancer are beneficial or antagonistic; this may be dependent on the tumor immunogenicity, the stage of disease, and the impact of inflammation and angiogenesis on tumor pathogenesis [65].

Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells and myeloid progenitor cells. MDSCs inhibit T cells activation [66, 67] in a nonspecific or antigen-specific manner, alter the peptide presenting ability of MHC class I molecules on tumor cells [68], influence B cells [69], block NK cell cytotoxicity [70–72], inhibit dendritic cell differentiation [73], and expand Tregs [74, 75] signifying their crucial contribution in constituting a tumor

suppressive environment. Furthermore, there is compelling evidence that MDSC, by secreting MMP9 and TGF-β1, are also involved in angiogenesis, vasculogenesis, and metastatic spread [76]. MDSCs suppress the immune system by the production of reactive oxygen species (ROS), nitric oxide (NO), peroxynitrite and secretion of the cytokines IL-10 and TGF-β [77]. Upregulated arginase-I activity by MDSCs depletes the essential amino acid L-arginine, contributing to the induction of T cell tolerance by the down regulation of the CD3ζ chain expression of the T cell receptor [78–81]. However, the mechanisms that are used to suppress the immune responses are highly dependent on the context of the tumor microenvironment [82]. An increased subpopulation of MDSCs in the peripheral blood of NSCLC patients was detected that decreased in those patients that responded to chemotherapy and patient undergoing surgery [83].

Tumor–Associated Macrophages

Macrophages are part of the innate immune system and play important roles in the first line of defense against foreign pathogens. They can be divided into M1 macrophages (classical activation) and M2 macrophages (alternative activation). M1 macrophages attract and activate cells of the adaptive immune system and have anti-tumor and tissue destructive activity, while the M2 phenotype has been linked to tumor-promoting activities by subversion of adaptive immunity, promoting tumor angiogenesis and supporting cancer cell survival, proliferation, invasion and tumor dissemination. Macrophages in tumors are usually referred to as tumor-associated macrophages (TAM) and their presence can be substantial (10–65 % of the tumor stroma). In the beginning, the TAM mainly consist of M1-like macrophages however, when the tumor starts to invade and vascularize, there is a skewing towards the M2 phenotype [84, 85]. This takes place especially at those regions in the tumor that are hypoxic [86]. It has been reported by several groups that there is an association between the number of tumor islet macrophages and NSCLC survival [87–91]. Moreover, when looking at the different phenotypes of TAM (M1 and M2), it is shown that high numbers of M1 macrophages infiltrating the tumor are correlated with improved survival. On the other hand, the presence of M2-like macrophages is associated with poor clinical outcome.

Dendritic Cells

Dendritic cells (DC) are widely acknowledged as the central surveillance cell type and play an important role in the activation of lymphocyte subsets to control or eliminate human tumors. Upon encountering tumor cells or tumor-associated antigens, DC engulf this material and begin migrating via lymphatic vessels to regional lymphoid organs. The density of immature DC (Langerhans cell and interstitial DC)

and mature DC, present in the tumor microenvironment, is highly predictive of disease-specific survival in early-stage NSCLC patients [92] and the presence of DC in resected NSCLC material is a good prognostic factor [9]. Interaction between the DC and tumor cells results in the release of anti-tumor cytokines [93, 94]. This suggests that DC within the tumor microenvironment of early-stage NSCLC are capable in initiating adaptive immune responses in situ [95–97]. In the peripheral blood and regional lymph nodes of lung cancer patients, the number and function of mature DC is dramatically reduced [98, 99], partly due to abnormal differentiation of myeloid cells (e.g. MDSC) [100]. Tumor cells, stromal cells like fibroblasts, and tumor-infiltrating immune cells and/or their secreted products, like VEGF, M-CSF, IL-6, IL-10, and TGF-β are also responsible for systemic and local DC defects [101–104]. Affected DC are impaired in their ability to phagocytose antigen and to stimulate T cells, leading to a defective induction of anti-tumor responses. NSCLC-derived DC produce high amounts of the immunosuppressive cytokines IL-10 and TGF-β [105]. It has been shown that the T cell co-inhibitory molecule B7-H3 and programmed death receptor-ligand-1 (PD-L1) are upregulated on tumor residing DC and these molecules conveys mainly suppressive signals by inhibiting cytokine production and T cell proliferation [106, 107].

Context-Specific Nature of Immune Cells in Lung Tumors

From the above it is apparent that different types of tumor-infiltrating immune cells have different effects on tumor progression. Based on their functions, immune types can be divided into cells with a potentially positive impact on the antitumor response and cells with detrimental effects, but cell phenotypes can adapt on the changing environment (e.g. the macrophage M1/M2 phenotype polarization). The net effect of the interactions between all these various cell types and their secreted products within the environment of an established tumor participates in determining anti-tumor immunity, angiogenesis, metastasis, overall cancer cell survival and proliferation. These immune infiltrates are heterogeneous between tumor types, and are very diverse from patient to patient. The importance of these tumor-infiltrating immune cells for tumorigenesis, and their secreted chemokines and cytokines, was recently acknowledged by revisiting the hallmarks of cancer as described by Hanahan and Weinberg in 2000 and now include “tumor promoting inflammation” and “avoiding immune destruction” [108, 109]. Besides the type and density of the cells, also the location in the tumor (in the centre or core; or within the invasive margins of the tumor), in adjacent tertiary or secondary lymphoid structures or their presence in peripheral blood will shape the immune contexture. Histopathological analyses of the location, density and functional orientation of the different immune cell populations in large annotated collections of human lung tumors has identified a good association of effector T-cells (CD3+CD8+), memory T cells (CD3+CD45RO+) [38, 88, 110–112], and Th1 cells (IL-2 and IFN-gamma secreting CD3+ cells) [110] with longer disease-free survival and/or a better overall

survival while Th17 [64, 110] and Tregs [56, 110, 113, 114] have a poor association with the prognosis [115, 116] . However, it is important to realize the complex spatiotemporal dynamics in the tumor-immune interactions in time due to perturbations at the gene and protein level of the immune cells and tumor cells within the microenvironment [116]. For example, when myeloid cells are attracted to the tumor, they are influenced by several signals able to shape the new cells as "needed" by the tumor. In an early phase of tumor development, the tumor-associated macrophages mainly consist of an M1-like phenotype and later in the tumorigenic process, when the tumor changes its local environment, there is a skewing towards the M2 phenotype [84, 85, 117]. This takes place especially at those regions in the tumor that are hypoxic (Fig. 2) [86, 118]. A subpopulation of TAMs gather in hypoxic sites in the tumor as a result of chemoattractants produced by tumor cells [119]. Exposure to hypoxia stimulates TAMs to acquire a pro-angiogenic M2 phenotype with high production of pro-angiogenic factors like VEGF and MMP-9 [120]. This preferential polarization is also a result of the absence of M1-orienting signals, such as IFN-γ or bacterial components in the tumor environment as well as the presence of M2 polarization factors. Thus the cancer immunoediting concept as described by Schreiber et al. [121], that comprises editing, equilibrium and escape, takes place continuously and this plasticity is a major source of heterogeneity between patients and a cause for therapy resistance [4].

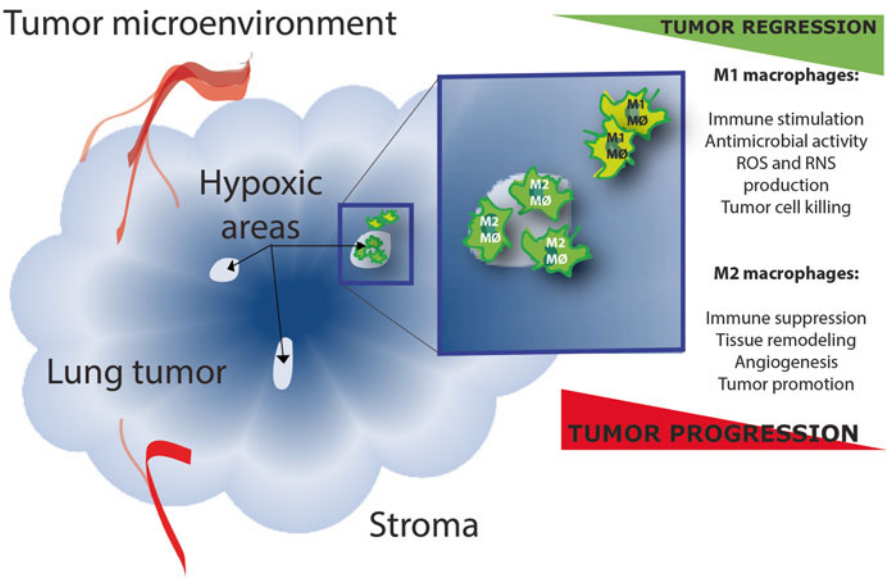

Fig. 2 Opposing effects of M1 and M2 tumor-associated macrophages (TAMs) in the tumor environment. ROS: reactive oxygen species and RNS: reactive nitrogen species. The classically activated M1 macrophages and alternatively activated M2 macrophages are at the opposing ends of the polarization continuum and have opposing effects in tumor biology. M2 TAMs can be abundantly present within tumors, especially in those areas that are oxygen deprived

Types of Immunotherapeutic Approaches

Immunotherapy attempts to stimulate or restore the body's natural ability of the immune system to fight cancer. There are various strategies to activate the immune system and these are classified earlier by Aerts et al. and here into the following categories: biological response modifiers, monoclonal antibodies, peptide or tumor cell vaccines, and cellular immunotherapy (Fig. 3 and Table 1). There is no consensus regarding which of the four categories is the optimal approach for thoracic malignancies, this will probably be highly dependent on the tumor characteristics of each individual patient.

Biological Response Modifiers

Biological response modifiers are compounds which can nonspecifically enhance the immune response, either by directly stimulating the immune system and/or by the direct induction of tumor cell apoptosis. These compounds can activate the anti-tumor immune response via the direct stimulation of pro-inflammatory immune

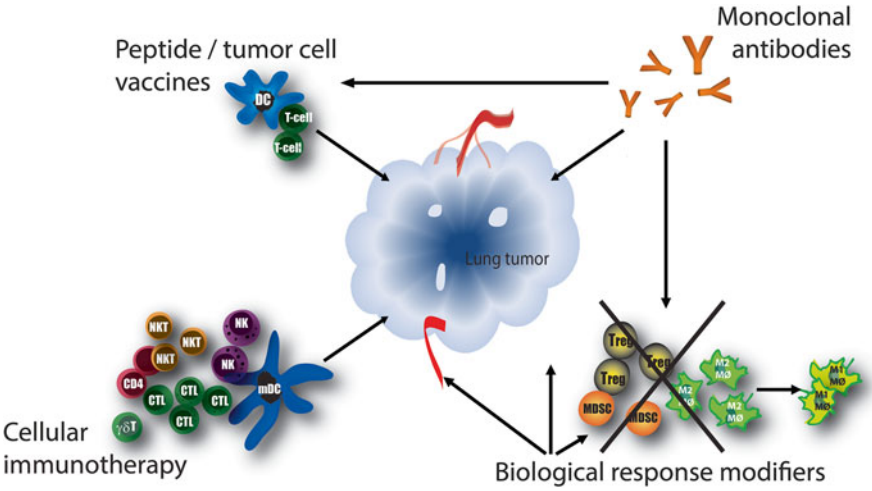

Fig. 3 Immunotherapeutic approaches. Different immunotherapeutic approaches are currently being developed for the treatment of lung cancer. All approaches aim to elicit an anti-tumor immune response and they can often work complementary and/or synergistically. Biological response modifiers can nonspecifically enhance the immune response, either by directly stimulating the immune system and/or by the direct induction of tumor cell apoptosis. Monoclonal antibodies bind specifically to one epitope and can be directed against numerous tumor- or immune cell related targets. Tumor vaccines are designed to deliver tumor antigens to antigen-presenting cells, which can subsequently induce a tumor specific immune response. Cellular immunotherapy includes the adoptive transfer of autologous or allogeneic activated immune cells

Table 1 Immunotherapeutic approaches that have been tested or are currently under investigation for lung cancer and mesothelioma

Approach types	Examples of clinical studies in lung cancer and mesothelioma	Mode of action	References
Biological response modifiers			
Triggering inflammation	PF-3512676 (CpG 7909) CpG-ODN 2006 Bacillus Calmette-Guerin (BCG), Mycobacterium vaccae (SRL172)	Toll-like receptor 9 agonist Downregulation of Tregs Nonspecific immune stimulants now often tested as adjuvants	[130, 131, 216, 217] [218] [219]
Cytokine therapy	IL-2 + tumor necrosis factor alpha (TNF-α) or interferon alpha (IFN-α) Interferon gamma (IFN-γ) Mda-7 (IL-24)	Induces T-cell proliferation Induces tumor cell apoptosis Mda-7/IL-24 induces tumor cell apoptosis and inhibits tumor angiogenesis	[135, 139] [136, 137] [220]
Colony-stimulating factors	Granulocyte colony-stimulating factor (G-CSF)	Treatment of chemotherapy-induced neutropenia	[140, 141]
Multi-modal effectors	Multi-target VEGFR: thalidomide and analogues such as lenalidomide and pomalidomide Cyclophosphamide Cyclosporine Denileukin Diftitox Talactoferrin Trabectedin (Yondelis) All-trans-retinoic acid (ATRA)	Besides anti-metastatic, anti-angiogenic also immunomodulatory drugs [221, 222] Targets regulatory T-cells [223, 224] Targets regulatory T-cells [225] Targets regulatory T-cells [226] and cancer cells which express the IL-2 receptor Recombinant human lactoferrin, promotes innate and adaptive immunity against tumor cells in the gut-associated lymphoid tissue [142, 227] Targets tumor-associated macrophages [228] and tumor cells Targets myeloid-derived suppressor cells (MDSCs)	[229, 230] [223] [231] [232] [143, 144, 233, 234] [235, 236] [237]

(continued)

Table 1 (continued)

Monoclonal antibodies			
Directed against tumor cells	Cetuximab Panitumumab Matuzumab Necitumab Trastuzumab (Herceptin) CAT-5001 (SS1P) Amatuximab (MORab-009)	Chimeric or fully humanized antibodies which target the epidermal growth factor (EGF) receptor on tumor cells Anti-HER2, targets tumor cells which overexpress the human epidermal growth factor 2 (HER2) protein Anti-mesothelin immunotoxin, targets mesothelin expressed in malignant mesothelioma and lung adenocarcinoma Chimeric anti-mesothelin monocloncal antibody	[145, 238, 239] [146, 240, 241] [242] [149]
Directed against tumor products	Bevacizumab	Slows the growth of tumor blood vessels by targeting the VEGF protein. Blockade of VEGF is also immunomodulatory	[150, 243]
Immune checkpoint inhibitors	Anti-CTLA-4 (Ipilimumab/tremelimumab) Anti-PD-L1 (BMS-936559/MPDL-3280A) / PD-1 (BMS-936558 (nivolumab)/MK3475 (lambrolizumab))	Prevents T cell inhibitory mechanisms and allows T cells to continue cancer cell destruction	[155, 244] [158, 159]
Peptide or tumor cell vaccines			

Vaccines	GVAX	GM-CSF gene-transfected tumor cell vaccine	[184, 185]
	Belagenpumatucel-L (Lucanix)	Allogeneic tumor cell vaccine made with four irradiated NSCLC cell lines modified with TGF-β2 antisense plasmid	[188, 247]
	MAGE-A3 vaccine	Vaccine composed of MAGE-A3 protein and adjuvant AS15	[166]
	(L)-BLP-25 anti-MUC-1 (Stimuvax)	Vaccine which targets MUC-1 expressed on tumor cells	[177, 248]
	TG4010	*Vaccinia* vector coding MUC1 and IL-2	[179, 180]
	CimaVax EGF	Vaccine composed of human recombinant Epidermal Growth Factor (EGF) conjugated to a carrier protein	[168]
	WT1 peptide vaccine	Vaccine composed of four WT1 (Wilms' tumor suppressor gene) analogue peptides	[169]
	CRS-207	Live-attenuated *Listeria monocytogenes* vector encoding human mesothelin [148]	[182]
	Bec2/BCG	Induces anti-GD3 antibodies (overexpressed on 60 % of SCLC patients) [245]	[124, 249]
	GV1001	Vaccine which targets the telomerase peptide GV1001	[173]
	Racotumomab (Vaxira)	Anti-idiotypic antibody which mimicks the NGcGM3 ganglioside that is expressed on multiple human cancers [246]	[250], ClinicalTrials.gov: NCT01460472
	Tergenpumatucel-L	Vaccine composed of irradiated and gene-transfected lung cancer cell lines	ClinicalTrials.gov: NCT01774578
Cellular immunotherapy			
Dendritic cells (DCs)	Ex-vivo generated DC-vaccines	Dendritic cells loaded with tumor antigens	[195, 254–256]
T-cells	Ex-vivo generated lymphokine-activated killer cells (LAK)	Autologous lymphokine-activated killer cells [251]	[196, 197]
Natural Killer (NK) cells	Cytokine-induced killer cells (CIK)	Autologous cytokine-activated T-cells and NK cells [252]	[200]
	Activated T-cells	Adoptive transfer of activated T-lymphocytes	[201]
	Gamma delta T cells	Adoptive transfer of zoledronate expanded gamma delta T-cells	[203]
	NK cells	Adoptive transfer of allogeneic Natural Killer (NK) cells [253]	[204]

cells or via the inhibition of detrimental suppressive immune cells like Tregs or MDSCs.

The observation that lung cancer patients who developed an empyema after pneumonectomy seemed to have a longer survival gave rise to studies involving different biological response modifiers in the 1970s [122]. The idea that bacterial infection in the area of the draining lymph nodes of the resected tumor could lead to immune destruction of residual tumor cells provoked studies involving the intrapleural injection of bacterial antigens to induce immune activation. Bacillus Calmette-Guèrin (BCG) is a vaccine against tuberculosis that is prepared from a strain of attenuated live bovine tuberculosis bacillus and its potential for cancer immunotherapy has been thoroughly investigated. McKneally et al. were the first to study the effect of postoperative injection of BCG into the pleural space of early stage lung cancer patients [123]. Their observation that intrapleural BCG injection resulted in an improved survival lead to numerous studies regarding nonspecific immune stimulation with this vaccine. Currently, BCG is most often investigated as an adjuvants instead as a single therapeutic agent in lung cancer patients [124]. In contrast, in patients with superficial bladder cancer, the use of intravesical BCG is now well-established [125]. In addition to BCG, heat-killed mycobacterium vaccae (SRL 172) has been investigated as a nonspecific immunostimulant in lung cancer and mesothelioma patients in combination with chemotherapy [126, 127], unfortunately no survival benefits were reported. Mycobacterial adjuvant-based agents have been shown to activate antigen-presenting cells and induce a Th1-type immune response, partly due to the binding of components of the cell wall of Mycobacteria to Toll-like receptors (TLRs) [128]. TLRs are membrane glycoproteins and belong to a family of pattern recognition receptors (PRRs) that recognize specific microbial molecular structures, pathogen associated molecular patterns (PAMPs). Recognition of a PAMP belonging to a micro-organism by a TLR leads to activation, maturation and induction of proinflammatory cytokines. Immature dendritic cells express numerous TLRs and nonspecific immune activation via the stimulation of these TLRs has been extensively researched. In lung cancer specifically, TLR9 plays an important role and has been described to be overexpressed in lung cancer tissue [129]. Synthetic TLR9-activating compounds (e.g. PF-3512676, CpG-ODN) have been clinically tested in combination with chemotherapy in lung cancer patients, unfortunately no clinical benefit was found [130, 131]. However, since preclinical studies have shown that the use of the TLR9 agonist CpG-ODN as an adjuvants in tumor vaccines reduces the number of regulatory T-cells and increases the number of effector T-cells, TLRs remain a potential target in the field of cancer immunotherapy [132].

In addition to compounds that nonspecifically enhance inflammation, the administration of cytokines has been amongst the earliest approaches in cancer immunotherapy. Interferons (IFN) have been one of the major cytokine families of interest given their direct antiproliferative and immunopotentiating effects. In the 1980s, the first clinical trials were conducted in which lung cancer patients were treated with different types of IFN (recombinant alpha and beta) [133, 134]. Since then, the potential of IFN therapy in lung cancer patients has been researched extensively in

a number of clinical trials, however no clinical benefits were found [135–138]. Other proinflammatory cytokines of which their potential as a therapeutic target in lung cancer patients has been investigated are IL-2 and TNF-α. In general, treatment with the combination of IL-2 and TNF-α induced relatively grave toxicities and no survival benefits [139]. Currently, the direct administration of proinflammatory cytokines in order to enhance the anti-tumor immune response has been mostly abandoned in lung cancer patients with the exception of the use of colony-stimulating factors with the purpose of the treatment of chemotherapy-induced neutropenia [140, 141].

The group of biological response modifiers consists of a subgroup of multi-modal effectors. The majority of the multi-modal effectors aim to enhance the anti-tumor immune response via the modulation of specific anti-inflammatory immune cells, e.g. Tregs, MDSCs and tumor-associated macrophages. Because of their capability to facilitate an optimal pro-inflammatory immune response, these multi-modal effectors have great potential in combination with more specific immunotherapeutic approaches, e.g. vaccines and cellular immunotherapy. A multi-modal effector which received a lot of attention recently is the recombinant human lactoferrin: talactoferrin alfa. Talactoferrin alfa is an immunostimulatory protein that stimulates dendritic cell maturation in the gut [142]. After promising preclinical and clinical studies in lung cancer patients, a phase 3 study showed no survival benefit of talactoferrin alfa monotherapy in patients with advanced lung cancer [143, 144]. However, the potential of this compound in combination with other immunotherapeutic strategies is topic of further research.

Monoclonal Antibodies

Monoclonal antibodies bind specifically to one epitope and their application as potential immunotherapeutic agents has received a lot of attention recently. The use of monoclonal antibodies directed against tumor growth related antigens on the tumor cell like epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) has been well established in lung cancer patients [145, 146]. In addition to the direct effect of the inhibition of growth factors and/or their receptors, antibodies bound to the tumor cell surface can induce antibody-dependent cell-mediated cytotoxicity (ADCC) [147]. Mesothelin is another tumor-specific antigen which is an attractive target for treatment with monoclonal antibodies because of its expression on several epithelial tumors including mesothelioma and lung cancer. Clinical studies with monoclonal antibodies against mesothelin are currently ongoing in lung cancer and mesothelioma patients [148, 149].

In addition to monoclonal antibodies directed against antigens specifically expressed by tumor cells, antibodies that are directed against tumor products have been clinically implemented. In lung cancer patients, the monoclonal antibody bevacizumab which is directed against VEGF has been extensively investigated in clinical trials. In a recent meta-analysis, Cui et al. showed that bevacizumab accom-

panied by chemotherapy improves clinical outcomes compared to other targeted therapies in chemotherapy-naïve lung cancer patients [150]. Bevacizumab has been developed in order to target blood vessel growth of tumors, however recent evidence shows that bevacizumab also has an immunomodulating effect and enhances circulating CD8 T cells in treated cancer patients [151]. This twofold effect makes bevacizumab an interesting compound to study in combination with other immunotherapies.

The blockade of immune checkpoints using monoclonal antibodies can be considered one of the major breakthroughs in cancer research of the past years. In order to control the immune response and to mitigate collateral tissue damage the immune system is harnessed with a negative feedback system. T-cells have the capacity to upregulate co-inhibitory receptors in order to inhibit the immune response and mediate immune tolerance. Multiple immune-inhibitory pathways (checkpoints) and their accompanying inhibitory co-receptors have been identified. In chronic infection and in cancer, expression of these inhibitory co-receptors is enhanced and associated with an anergic state in T cells [152]. Antibodies that bind to these co-receptors can block inhibitory signals and therefore augment T cell activation and proliferation.

The development of antibodies which bind to co-inhibitory molecules activated during T cell activation has led to the possibility to prevent T cell inhibitory mechanisms and therefore enhance the anti-tumor immune response [153]. The first monoclonal antibody against a co-inhibitory molecule that showed clinical efficacy in cancer patients was anti-CTLA-4 (ipilimumab) [3]. Currently, ipilimumab is approved for the treatment of metastatic melanoma [154]. In patients with advanced lung cancer, treatment with ipilimumab and chemotherapy resulted in a modest survival benefit compared to treatment with chemotherapy alone [155]. Another monoclonal antibody directed against CTLA-4, tremelimumab, has shown encouraging clinical activity in patients with chemotherapy-resistant advanced mesothelioma [156]. In addition to CTLA-4, expression of other co-inhibitory receptors like programmed death protein (PD1) and its ligand PD-L1 have been shown to play a role in lung cancer [157]. Both treatment with anti-PD1 (nivolumab) and anti-PD-L1 resulted in objective responses in patients with advanced lung cancer [158, 159]. Although these results are without any doubt very promising, the implementation of these immune checkpoint inhibitors is hampered by serious immune-related toxicities (e.g. colitis, hepatitis) and low response rates. Therefore, the development of robust, predictive biomarkers is pivotal for the clinical implementation of monoclonal antibodies against co-inhibitory receptors [160]. In addition, phase 3 studies will have to determine whether these immune checkpoint inhibitors will earn their place in the standard treatment of lung cancer.

Tumor Vaccines

The research regarding cancer vaccines has made great progress since the discovery of human tumor antigens which can be recognized by T cell receptors [161]. Tumor vaccines are designed to deliver tumor antigens to antigen-presenting cells, which can subsequently induce a tumor specific immune response by the adaptive immune

system. These vaccines can consist of various types of antigen sources. An antigen candidate needs to meet certain criteria in order to potentially be able to elicit a specific anti-tumor immune response. Tumor specificity, frequency and homogeneous expression in tumor cells, role as an oncogene and intrinsic immunogenicity are essential features of antigens which determine the success [5]. In lung cancer and mesothelioma, a broad spectrum of approaches using various antigen sources have been undertaken to develop cancer vaccines. These are here divided into (1) proteins and peptides, (2) liposomal complexes, (3) recombinant viruses and bacterial vectors, and (4) cell-based vaccines.

Proteins and Peptides

Melanoma-associated antigen A3 (MAGE A3) is an antigen which is specifically expressed by several human tumors, including non-small cell lung cancer (NSCLC). Activation of the MAGE genes is known to take place in early carcinogenesis of the lung, however the physiological function of MAGE gene products is unknown [162, 163]. MAGE A3 is detected in approximately 35–50 % of NSCLC and its expression has been shown to be inversely correlated with survival [164, 165]. Vaccines composed of recombinant MAGE A3 (and adjuvants) have shown promising results in lung cancer patients in phase 2 studies and have progressed to a phase 3 trial which is currently being conducted [166]. Epidermal growth factor (EGF) is another eligible candidate to be applied in lung cancer vaccines because of its broad expression in 85 % of NSCLCs. CimaVax EGF is a vaccine composed of human recombinant EGF conjugated to a carrier protein and has proven survival benefits in a phase 2 study with advanced-stage lung cancer patients [167, 168]. In addition to large proteins like MAGE and EGF, smaller peptides can also be used in tumor vaccines. The WT1 (Wilms' tumor suppressor gene 1) peptide vaccine is composed of four WT1 analogue peptides. WT1 can be expressed in both lung cancer and mesothelioma and vaccination with this peptide has been shown to induce T-cell mediated immune responses in lung cancer and mesothelioma patients [169]. The enzyme telomerase is expressed in most human cancers, including NSCLC, and is therefore considered an attractive cancer vaccine target [170, 171]. Peptide GV1001 consists of 16 amino acids derived from the active site of human telomerase reverse transcriptase [172]. A phase 2 trial with GV1001 showed low toxicity, immune activation and promising clinical responses in NSCLC patients [173].

Liposomal Complexes

Liposomes are known to be potent vaccine delivery systems [174]. The best known cancer vaccine which makes use of this delivery technique is L-BLP25 or Stimuvax. This liposomal vaccine aims to generate an immune response against mucin 1 (MUC1), a cell surface glycosylated phosphoprotein that is frequently overexpressed by epithelial tumors including NSCLC [175, 176]. The L-BLP25 contains

the BLP25 lipopeptide and a liposomal delivery system, which facilitates uptake by antigen-presenting cells [176]. A phase 2 trial with advanced stage NSCLC patients showed survival benefits and paved the way for a large phase 3 trial (the START trial) [177]. Unfortunately, Stimuvax failed to increase overall survival in this trial, however, subgroup analyses could still reveal beneficial effects for certain lung cancer patient groups and/or treatment schemes [178].

Recombinant Viruses and Bacterial Vectors

Viruses can be genetically modified in order to express certain antigens and/or co-stimulatory cytokines and are therefore useful as 'viral vaccines' in cancer immunotherapy. The earlier described MUC1 protein can also be targeted by the TG4010 viral vaccine. This vaccine consists of attenuated vaccinia virus Ankara which is genetically modified to express MUC1 and IL-2 as adjuvants [179]. In a phase 2 study it was shown that TG4010 enhances the effect of chemotherapy in advanced NSCLC patients, a phase 3 trial is currently being conducted [180].

In addition to viruses, bacteria can also be used as a vaccination vehicle. CRS-207 consists of a live-attenuated *Listeria monocytogenes* vector encoding human mesothelin [148]. The earlier described mesothelin is an attractive vaccine component because it has been shown to be able to induce a strong CD8 T cell response [181]. CRS-207 has proven to be safe and resulted in immune activation in a phase 1 study amongst patients with mesothelin-positive tumors (lung cancer and mesothelioma included) [182]. Future studies will have to reveal the clinical potential of this mesothelin encoding bacterial vector.

Cell-Based Vaccines

There is a variety of cell-based vaccines under development for the treatment of lung cancer. Cell-based vaccines can be autologous or allogeneic and transfected or not with immunostimulatory compounds. Autologous tumor cell vaccines are ideal antigen sources because they are capable of inducing an immune response to a large variety of antigens expressed by the patient's tumor. However their practical implementation is complex and challenging for large scale development [183]. An example of an autologous tumor cell vaccine developed for lung cancer is GVAX. This autologous lung cancer vaccine consists of patient-specific irradiated lung cancer cells genetically modified to secrete granulocyte-macrophage colony stimulating factor (GM-CSF) to enhance the immune response. Although a phase 1 study with GVAX showed signs of clinical benefits in lung cancer patients, this study was not followed-up by a phase 2 or phase 3 study [184, 185]. An allogeneic tumor cell vaccine that did reach phase 3 clinical trials is belagenpumatucel-L (Lucanix). Lucanix consists of four irradiated NSCLC cell lines modified with TGF-β2 antisense plasmid. TGF-β is known to be associated with the immune escape of tumors and increased levels of TGF-β are associated with a worse prognosis in NSCLC patients

[186, 187]. The addition of the TGF-β2 antisense plasmid aims to stimulate the vaccine-induced immune response by inhibition of the production of TGF-β by the tumor. It is possible to use a combination of tumor cell lines as vaccine cocktail because NSCLC tumor cell lines are described to share immunogenic epitopes with primary tumors [163]. A phase 2 study showed clinical response rates of 15 % amongst advanced stage NSCLC patients [188]. Unfortunately, in a phase 3 study belagenpumatucel-L did not meet its predefined endpoint in the entire patient population. However, in specific subgroups of patients marked improvements in survival were achieved resulting in a current continued development of belagenpumatucel-L for specific indications.

Cellular Immunotherapy

Cellular immunotherapy includes the adoptive transfer of autologous or allogeneic activated immune cells. Initially, adoptive immunotherapy was used for relapses after allogeneic bone marrow transplantation in leukemia patients [189]. Recent advances have facilitated the application and clinical success of this method in various solid tumors [190]. The most prominent success story regarding cellular immunotherapy is sipuleucel-T, a vaccine for prostate cancer that consists of autologous peripheral blood mononuclear cells (PBMCs) including antigen-presenting cells that have been activated ex vivo with a recombinant fusion protein (PA2024, a prostate antigen that is fused to GM-CSF) [2]. After it was demonstrated in a phase 3 clinical trial that sipuleucel-T prolongs survival in metastatic castration-resistant prostate cancer patients, FDA approval followed in 2010. The general goal of adoptive cellular immunotherapy is to induce a tumor-specific immune response via the infusion of e.g. tumor-antigen loaded DCs or specifically activated T cells. In lung cancer, cellular immunotherapeutic approaches using various cell types have been evaluated [191].

Dendritic Cells

As described earlier, DCs are the professional antigen-presenting cells of the immune system and they have emerged as the most powerful initiators of immune responses. Because of their capacity to engulf tumor antigens and activate T-cells in an antigen-specific manner, the use of DCs as immunotherapeutic agents is very promising. In DC-based immunotherapeutic approaches, DCs are generated ex vivo from monocytes and after arming with tumor-associated antigens, reinjected into the patient with the intention to restore proper presentation of tumor-associated antigens and T-cell activation. This concept has been researched in NSCLC and has shown promising results regarding the elicited immune response, safety and tolerability, despite the small sample sizes of the trials [192–194]. In mesothelioma, treatment with autologous tumor-lysate pulsed DCs was shown to be safe and elicited an anti-tumor immune response in a phase 1 clinical trial [195].

T Cells

Different sources and activation procedures can be used in specifically harnessing the T cell response and have been clinically evaluated in lung cancer. Lymphokine-activated killer (LAK) cells are autologous IL-2 stimulated lymphocytes and their application has shown clinical responses in several studies with lung cancer patients [196, 197]. Cytokine-induced killer (CIK) cells are generated in vitro by stimulation of peripheral blood lymphocytes with anti-CD3 antibodies, IL-2, IL-1α, and IFN-γ [198]. CIK cells have proven their clinical potential in multiple solid tumors including lung cancer [199] and these results warrant future clinical trials [200]. In addition, in a recent study autologous purified T lymphocytes activated with anti-CD3 antibodies and IL-2 demonstrated an extended survival in patients with advanced NSCLC, however this effect was seen in a historical cohort study and therefore these data will have to be confirmed in a prospective randomized trial [201]. Because expanded gamma delta T cells showed strong cytotoxicity to lung cancer cell lines in vitro, the exploitation of this cell type has been tested clinically. In a phase 1 study, autologous expanded gamma delta T cells unfortunately showed limited clinical responses in NSCLC patients [202, 203].

Natural Killer Cells

Adoptive transfer of allogeneic, in vitro activated and expanded NK cells from haploidentical donors was proven potentially clinically effective in NSCLC [204]. NKT cells are currently exploited for cancer treatment by harnessing these cells with CD1d agonist ligands [205], or by adoptive transfer of NKT cells activated in vitro [206].

Response Evaluation

Immunotherapy represents a new class of agents in the treatment of lung cancer. As demonstrated for sipuleucel-T in prostate cancer and ipilimumab in melanoma, treatment responses and improvement of overall survival can be seen in lung cancer patients. However, often the agents did not change initial disease progression. Even a transient worsening of disease manifested either by progression of known lesions or the appearance of new lesions can be seen, before disease stabilization or tumor regression. The commonly accepted treatment paradigm, however, suggests that treatments should initially decrease tumor volume, which can be measured using CT scan. Also, progression-free survival is increasingly used as an alternative endpoint of studies. This seems to be unfortunate for immunotherapy, which may initiate an immune response that ultimately slows the tumor growth rate, resulting in longer survival, but not a decrease in tumor volume on CT scan or an increased progression-free survival [257]. Immune-related response criteria which adapt the standard response criteria and include the potential for delayed clinical response

and initial increase of tumor mass after immunotherapy are currently being developed [207]. Therefore, clinicians at this moment may need to reconsider how to measure success of their immunotherapeutic approach [208].

The Future of Cancer Immunotherapy

For a long time, stimulating the patient's immune system to attack tumors has been viewed as a rather meaningless intervention, an assumption that has radically changed since the recent clinical successes of cancer immunotherapy. However, despite the promising results achieved in some patients, the overall response rates are low. It has been shown that the immune contexture (i.e., the type, density and location of tumor-infiltrating immune cells) can predict the clinical outcome of patients affected by multiple types of cancer, but with consistent intra-patient variations [209]. It is therefore critical to identify the unique immunological profile of individual patients as a means to identify the best-suited immunotherapeutic approach for each patient [153]. This personalized way could greatly improve the efficacy of current immunotherapies. In addition to an individualized treatment plan, the future of immunotherapy in lung cancer patients includes multimodality treatment. Surgery, irradiation or chemotherapy vigorously reduce the tumor mass and the induced tumor cell death results in tumor antigen exposure. These therapeutic effects enhance the efficacy of immunotherapy and warrant combinatorial treatment approaches. In addition, multiple chemotherapeutical agents have been described to modulate the tumor microenvironment and therefore enhance immunotherapy [210, 211]. Gemcitabine and vinorelbine are known to be able to reduce circulating MDSCs [212, 213], increase the ratio of M1 to M2 macrophages [214] and stimulate APCs [215]. Therefore, the combination of immunotherapy with conventional treatments can elicit a synergistic treatment response and takes advantage of the full potential of cancer immunotherapy.

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Anti-angiogenesis in Personalized Therapy of Lung Cancer

Peter M. Ellis

Abstract Upregulation of angiogenesis is a frequent occurrence in lung cancer and is reported to represent a negative prognostic factor. This provides a rationale for the development and evaluation of anti-angiogenic agents. To date bevacizumab, a monoclonal antibody directed against serum VEGF, is the only anti-angiogenic agent that has demonstrated improved overall survival for patients with lung cancer. Meta-analysis of trials of bevacizumab in combination with platinum-based chemotherapy for NSCLC, show a 10 % reduction in the risk of death (HR 0.90, 95 % CI 0.81–0.99). However, therapy with bevacizumab is limited to NSCLC patients with non-squamous histology, good performance status, no brain metastases and the absence of bleeding or thrombotic disorders. More recently, similar survival was observed in a non bevacizumab containing regimen of carboplatin, pemetrexed and maintenance pemetrexed.

Multiple oral anti-angiogenic compounds have been evaluated in NSCLC, both in first-line therapy, or upon disease progression. The majority of agents have shown some evidence of activity, but none have clearly demonstrated improvements in overall survival. Increased toxicities have been observed, including an increased risk of death for some agents, limiting their development. Promising data exist for sunitinib in patients with heavily pre-treated NSCLC, and nintedanib in combination with docetaxel, as second-line therapy for NSCLC. However, these findings require validation. Currently, there is no established role for anti-angiogenic therapy in SCLC, although there is some promise for sunitinib as maintenance therapy following platinum and etoposide chemotherapy.

The challenge for anti-angiogenic therapy is to understand whether treatment effects in a subpopulation, are lost among a larger unselected population of patients. There is a need for additional translational research to identify predictive biomarkers for anti-angiogenic therapy.

Keywords Anti-angiogenic therapy • NSCLC • SCLC • VEGFR • PDGFR • FGFR • Bevacizumab • VEGF-trap • Tyrosine kinase inhibitors • Predictive biomarkers

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Rationale for Anti-Angiogenesis in Lung Cancer

Angiogenesis is a complex process occurring throughout the body and subject to many pro and anti-angiogenic regulatory factors. Beyond normal physiological function though, new blood vessel formation and upregulation of angiogenesis is frequently observed in many cancers, including lung cancer [1] (Fig. 1).

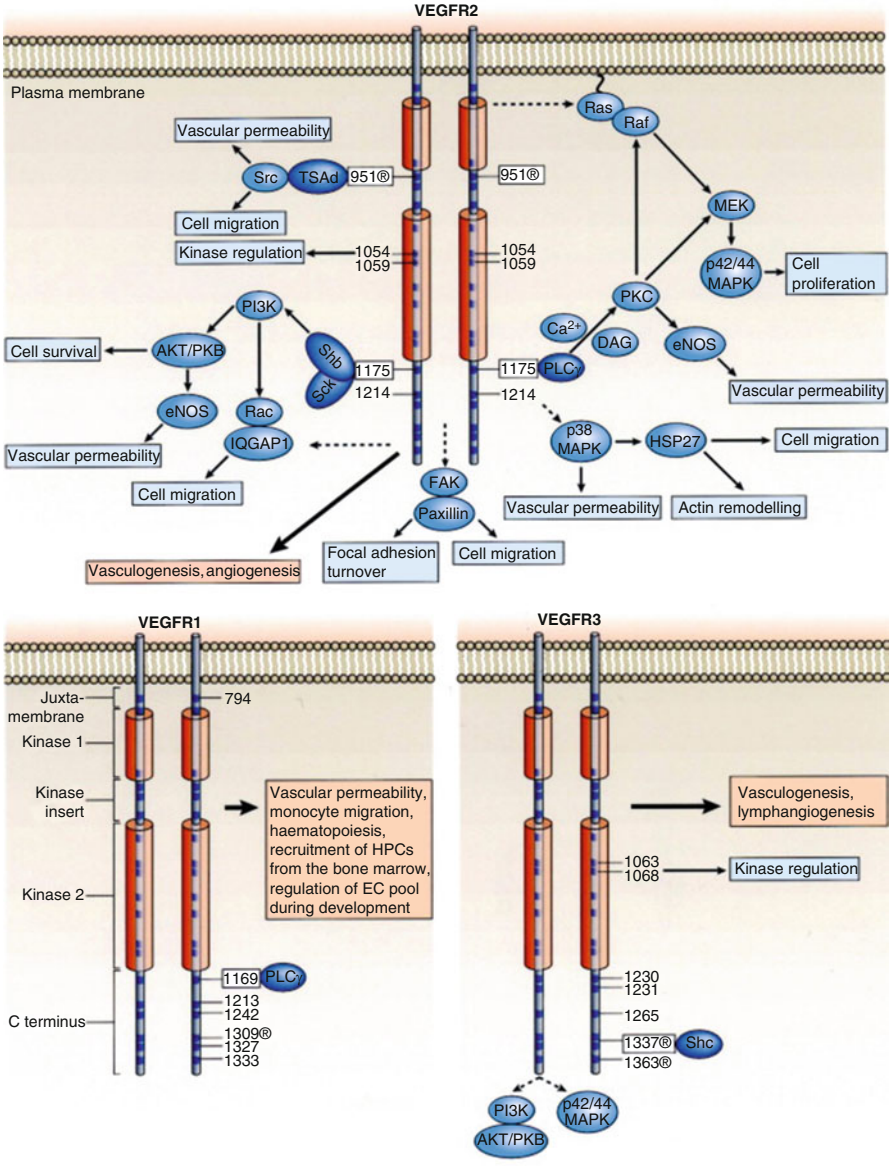

Fig. 1 Vascular endothelial growth factor receptor signal transduction (Reprinted with permission from Roskoski. CROH 2007 62(3):179–213)

A variety of pro-angiogenic factors exist, including vascular endothelial growth factor (VEGF), [2, 3] platelet derived growth factor (PDGF) [4] and fibroblast derived growth factor (FGF) [5]. Recognition of the importance of these factors in tumor growth and development has resulted in the development and evaluation of multiple therapeutic agents as potential anticancer therapies [6].

The role of serum VEGF (VEGF-A) in tumor growth and development has been widely studied. Serum VEGF activates the VEGF receptor (VEGFR-2) resulting in downstream signalling through activation of targets including PI3-kinase, resulting in stimulation of cell survival and proliferation pathways [7, 8]. Overexpression of VEGF-A appears to be a negative prognostic factor for survival [9]. Given its role in tumor progression and negative impact on survival, the VEGF/VEGF-R pathway has been the focus of extensive therapeutic evaluation for anti-angiogenic agents.

The Platelet Derived Growth Factor (PDGF) family of receptors represent an additional pro-angiogenic pathway. PDGF results in stimulation of PDGF receptor α (PDGFR-α) and PDGF receptor β (PDGFR-β) [4, 10] resulting in downstream signalling through PI3K and extracellular signal-regulated kinase (Erk) [11]. In vitro data suggest that PDGFR expression in tumors is a mechanism of resistance to VEGF directed therapy and provide a rationale for combined VEGF and PDGF directed therapy [12, 13].

Fibroblast Growth Factor (FGF) and its receptor represent a further anti-angiogenic target [14]. Similar to VEGF and PDGF, ligand binding causes receptor dimerization, activation of the tyrosine kinase domain, and stimulation of downstream targets, including PI3K and the mitogen-activated protein kinase (Mek)-Erk pathways [5]. In vitro data suggest synergism between FGF, VEGF, and PDGF pathways in stimulating angiogenesis and cellular growth [15, 16]. Resistance to anti-VEGF therapy may be due in part to upregulation of compensatory angiogenic signaling pathways (cross-talk), such as PDGF and FGF, therefore, inhibition of multiple pro-angiogenic pathways may represent a rational treatment strategy for patients with NSCLC.

These data provide a rationale for the evaluation of agents inhibiting VEGF, PDGF, or FGF as treatment options for lung cancer. Anti-angiogenic agents have been widely evaluated in combination with standard systemic therapies. There are several proposed mechanisms of action through which anti-angiogenic therapies can provide further benefit to the therapeutic effect of other systemic treatments [6]. Firstly these agents may normalize tumor vasculature and improve delivery of cytotoxic agents. Secondly, they may prevent rapid tumor cell repopulation after cytotoxic drugs. Lastly they may augment the anti-vascular effects of chemotherapy. At present, bevacizumab, a monoclonal antibody directed against VEGF-A, is the only anti-angiogenic agent approved as a treatment for lung cancer [17]. Additional strategies target receptor tyrosine kinases for VEGFR, PDGFR and FGFR, as well as vascular disrupting agents (Table 1). Clinical data for all these of agents are summarized in the following sections.

Table 1 Summary of mechanism of action for anti-angiogenic agents

Drug	VEGF	VEGFR	PDGFR	c-kit	Raf	Flt-3	RET	Pan-Her	MET	FGFR	EGFR	Src
Bevacizumab	✓											
Sorafenib		R-2&3	β	✓	✓	✓						
Sunitinib		R-1,2&3	α/β	✓		✓	✓					
Cediranib		R-1,2&3	α/β	✓								
Axitanib		R-1,2&3	β	✓								
Motesanib		R-1,2&3	✓	✓			✓					
Linifanib		R-1,2&3	✓									
BMS-690514		✓						✓				
MGCD265		✓							✓			
Brivanib		R-2								R-1		
Vandetanib		R-2&3					✓				✓	
BIBF1120		R-1,2&3	α/β			✓				R-1,2&3		✓
Pazopanib		R-1,2&3	α/β	✓						R-1,3		
Ramucirumab		R-2										
Aflibercept	VEGF-A&B											

The Role of Bevacizumab in the Treatment of NSCLC

Bevacizumab in the First-Line Therapy of NSCLC

Bevacizumab is a monoclonal antibody directed towards VEGF-A, and is the only anti-angiogenic agent to date, that has been shown to improve overall survival in lung cancer [17]. Toxicity concerns, including venous thromboembolic disease and fatal hemorrhage were observed in the initial randomized phase II trial [18]. This limited further development of bevacizumab in NSCLC to good performance status patients with non-squamous histology and no history of thrombosis, bleeding, gross hemoptysis, or brain metastases.

Five randomized trials have evaluated the addition of bevacizumab to standard platinum-based chemotherapy as first-line therapy for advanced and metastatic NSCLC (Table 2) [17–22]. The Eastern Cooperative Oncology Group (ECOG) 4599 trial randomized patients to carboplatin, paclitaxel with or without bevacizumab 15 mg/kg [17]. Greater efficacy was observed for patients receiving chemotherapy plus bevacizumab, including higher response rates (RR; 35 % vs. 15 %; $p<0.001$), longer progression free survival (PFS; 6.2 vs. 4.5 months; hazard ratio [HR], 0.66; 95 % confidence interval [CI], 0.57–0.77) and improved overall survival (OS; 12.3 vs. 10.3 months; HR, 0.79; 95 % CI, 0.67–0.92). The Avastin in Lung Cancer trial (AVAiL), evaluated the addition of two dose levels of bevacizumab (7.5 and 15 mg/kg) to cisplatin and gemcitabine chemotherapy. Significant improvements were observed in response rates and PFS for patients randomized to either dose of bevacizumab, compared with cisplatin and gemcitabine alone [20]. However, no improvement was observed in OS for patients randomized to bevacizumab 7.5 mg/kg (HR, 0.93; 95 % CI, 0.78–1.11), or 15 mg/kg (HR, 1.03; 95 % CI, 0.86–1.23) [21]. Two additional trials done in Japan and China provide confirmatory data regarding the efficacy of bevacizumab in combination with carboplatin and paclitaxel [19, 22]. Both demonstrate improvements in RR and PFS.

In all of these trials, bevacizumab was administered as maintenance therapy until disease progression. This strategy has not been formally evaluated, although retrospective review of data from both the ECOG 4599 trial and the US Oncology network suggests that patients continuing bevacizumab until disease progression experienced longer PFS and overall survival [23, 24].

Two meta-analyses of platinum-based chemotherapy with or without bevacizumab both report a significant improvement in OS for patients randomized to receive bevacizumab [25, 26]. Soria et al, report a 10 % reduction in the risk of death from the addition of bevacizumab to chemotherapy (HR 0.90, 95 % CI 0.81–0.99) [25]. Significant improvements were also observed in response rate and PFS [26]. However, the addition of bevacizumab to platinum-based chemotherapy is associated with increased toxicities. In the ECOG 4599 trial, patients randomized to chemotherapy plus bevacizumab experienced more grade 3–5 neutropenia ($p=0.002$), thrombocytopenia ($p=0.04$), febrile neutropenia ($p=0.02$), hyponatremia ($p=0.02$), hypertension ($p<0.001$), proteinuria ($p<0.001$), headache ($p=0.003$), rash/desquamation

Table 2 Summary of randomized trials evaluating the addition of bevacizumab to systemic therapy for NSCLC

Trial	Design	Intervention	Number	ORR	PFS	OS	QoL
First-line therapy							
Johnson [18]	Randomized phase II	Cb+Pac+Bev 7.5 mg/kg Cb+Pac+Bev 15 mg/kg Cb+Pac	35 32 32	31.5 % 28.1 % 18.8 %	7.4 m 4.3 m 4.2 m	17.7 m 11.6 m 14.9 m	NA
ECOG4599 [17]	Phase III	Cb+Pac+Bev 15 mg/kg Cb+Pac	434 444	35 % 15 % P<0.001	6.2 m 4.5 m HR 0.66 (0.57–0.77)	12.3 m 10.3 m HR 0.79 (0.67–0.92)	NA
AVAiL [20, 21]	Phase III	Cis-Gem+Bev 15 mg/kg Cis-Gem+Bev 7.5 mg/kg Cis-Gem+Plac	351 345 347	30.5 % 34.1 % 20.1 %	6.5 m (HR 0.82, p=0.03) 6.7 m HR (0.79, p=0.003) 6.1 m	13.4 m (HR 1.03, 0.86–1.23) 13.6 m (HR 0.93, 0.78–1.11) 13.1 m	NA
JO19907 [19]	Randomized Phase II	Cb+Pac+bev 15 mg/kg Cb+Pac	121 59	60.7 % 31 %	6.9 m 5.9 m (HR 0.61, 0.42–0.89)	22.8 m 23.4 m (HR 0.99, 0.65–1.50)	NA
BEYOND [22]	Phase III	Cb+Pac+Bev 15 mg/kg Cb+Pac+Plac	138 138	54.4 % 23.3 %	9.2 m 6.5 m (HR 0.40, 0.29–0.54)		NA
AVAPERL [33, 34]	Phase III	Cis+Pem+Bev 7.5 mg/kg then randomized to maintenance Bev 7.5 mg/kg+Pem Bev 7.5 mg/kg	376 128 125	22.7 %	7.4 m 3.7 m (HR 0.48, 0.35–0.66)	NR 12.8 m (HR 0.75, 0.47–1.19)	EORTC QLQ-30 and LC13. No difference in global QoL. Role function, fatigue and appetite favour bev. Pain favour bev + pem

Point Break [35]	Phase III	Cb+Pem+Bev 15 mg/kg → Bev+Pem Cb+Pac+Bev 15 mg/kg → Bev	472 467	34 % 33 %	6.0 m 5.6 m (HR 0.83, 0.71–0.96)	13.4 m 12.6 m (HR 1.0, 0.86–1.16)	NA
Pronounce [36]	Phase III	Cb+Pem → Pem Cb+Pac+Bev 15 mg/kg → Bev	182 179	23.6 % 27.4 %	G4PFS 3.9 m 2.9 m (HR 0.85, 0.70–1.04)	10.5 m 11.7 m (HR 1.07, 0.83–1.36)	NA
Second line therapy							
Herbst [40]	Randomized phase II	Doc/Pem+Bev 15 mg/kg Erlo+Bev 15 mg/kg Doc/Pem+Plac	40 39 41	12.5 % 17.9 % 12.2 %	4.8 m (HR 0.66, 0.38–1.16) 4.4 m (HR 0.72. 0.42–1.23) 3.0 m	12.6 m (HR 0.71, 0.41–1.21) 13.7 m (HR 0.78, 0.42–1.23) 8.6 m	NA
BeTa [39]	Phase III	Erl+Bev 15 mg/kg Erl+Plac	319 317	13 % 6 %	3.4 m 1.7 m (HR 0.62, 0.52–0.75)	9.3 m 9.2 m (HR 0.97, 0.80–1.18)	NA

Cb carboplatin, *Pac* paclitaxel, *Bev* bevacizumab, *NA* not assessed, *Cis* cisplatin, *Gem* gemcitabine, *Plac* placebo, *Pem* pemetrexed, *G4PFS* progression free survival without grade 4 toxicity, *Doc* docetaxel, *Erl* erlotinib

($p=0.02$), and bleeding events ($p<0.001$). There were 15 treatment-related deaths, including 5 due to pulmonary hemorrhage [17]. A similar profile of adverse events was observed in the AVAiL trial.

Given the toxicity concerns with bevacizumab, a variety of sub group analyses have been performed on these trials to identify subgroups of patients who derive greater benefit from bevacizumab therapy. In the ECOG 4599 trial, a larger improvement in overall survival was observed in patients with adenocarcinoma histology [27]. The median survival was 14.2 months compared with 10.3 months in the adenocarcinoma subgroup (HR 0.69, 95 %CI 0.58–0.83). Subgroup analyses of Asian patients in the AVAiL trial suggest that the addition of bevacizumab may improve survival in this group of patients [28]. Biomarkers including serum VEGF, intracellular adhesion molecule (ICAM), βFGF have all been evaluated as predictive biomarkers for bevacizumab. While ICAM was prognostic for improved survival and both ICAM and VEGF were predictive for response rates, no biomarker to date has been shown to predict a differential effect on OS for patients receiving bevacizumab. The onset of hypertension, a known side effect of bevacizumab, has also been evaluated as a predictive biomarker of efficacy [29]. However, a combined analysis of seven trials including disease sites other than lung cancer, did not support hypertension as a predictive biomarker. Therefore none of the biomarkers evaluated to date, are predictive for an improvement in overall survival from bevacizumab [30]. Therefore, it is not possible to further define subgroups of patients to receive bevacizumab therapy.

Competing therapeutic strategies to bevacizumab have emerged over recent years. Maintenance therapy with pemetrexed following initial platinum-doublet chemotherapy has been shown to improve survival in advanced NSCLC [31, 32]. Therefore, additional trials have tried to evaluate the role of pemetrexed, in bevacizumab eligible patients. Two trials have incorporated maintenance pemetrexed therapy in patients receiving bevacizumab. The AVAPERL trial randomized patients to maintenance therapy with bevacizumab alone (Table 2), or in combination with pemetrexed, following induction therapy with cisplatin, pemetrexed plus bevacizumab [33]. Three hundred and seventy six patients were registered and 253 randomized to maintenance therapy. PFS, the primary outcome, was prolonged in patients receiving maintenance bevacizumab plus pemetrexed compared with bevacizumab alone (7.4 m vs. 3.7 m HR 0.48, 95 %CI 0.35–0.66). AVAPERL failed to show a significant difference in overall survival as a secondary endpoint (HR 0.75, 95 %CI 0.47–1.19). Global quality of life (QoL), assessed by the EORTC QLQ-30, was similar between the two arms, although role function, fatigue and appetite favoured the combination of bevacizumab and pemetrexed [34]. Similarly, the Point Break trial evaluated the addition of pemetrexed to maintenance bevacizumab [35]. Patients were randomized to carboplatin, paclitaxel, bevacizumab then maintenance bevacizumab, versus carboplatin, pemetrexed, bevacizumab then maintenance pemetrexed plus bevacizumab. There was a modest improvement in PFS (6 m vs. 5.6 m, HR 0.83, 95 %CI 0.71–0.96), but no improvement in overall survival (13.4 m vs. 12.6 m, HR 1.0, 95 %CI 0.86–1.16). Maintenance therapy with pemetrexed in addition to bevacizumab should not be considered the standard of care at this time in the absence of improved overall survival.

One additional trial (Pronounce) compared carboplatin, paclitaxel plus bevacizumab with a non-bevacizumab regimen of carboplatin, pemetrexed then maintenance pemetrexed [36]. The primary outcome was a composite of progression free survival without grade 4 toxicity. Similar response rates, PFS and overall survival were observed between the two arms, suggesting that bevacizumab may not add to the efficacy of more effective chemotherapy. Currently, bevacizumab is widely used in combination with platinum-based chemotherapy and has been incorporated into NSCLC treatment algorithms [37]. It does have significant incremental toxicity and data from the Pronounce trial suggest that cisplatin or carboplatin plus pemetrexed followed by maintenance pemetrexed provides an alternative to bevacizumab based therapy.

Bevacizumab in the Second Line Therapy of Advanced NSCLC

There has been some interest in continuation of bevacizumab beyond progression, however, there are no data from randomized trials to support this. There is an ongoing trial (AVaALL) evaluating this strategy [38]. However, two trials have evaluated the addition of bevacizumab to erlotinib in the second-line setting [39, 40]. Herbst et al. [40], conducted a randomized phase II trial comparing the addition of bevacizumab to either second-line chemotherapy, or erlotinib. Response rates were highest with the combination of erlotinib plus bevacizumab (Table 2) and improvements in PFS and OS were seen with both bevacizumab containing arms. Subsequently, the phase III BeTa trial randomized patients to erlotinib plus bevacizumab versus erlotinib alone [39]. Significant improvements were observed in RR (13 % vs. 6 %) and PFS (3.4 m vs. 1.7 m, HR 0.62, 95 %CI 0.52–0.75). However, there was no improvement in overall survival (9.3 m vs. 9.2 m, HR 0.97, 95 %CI 0.80–1.18). As a result, there are no data currently supporting the use of bevacizumab as second-line therapy in combination with either erlotinib or chemotherapy.

Other Intravenous Anti-Angiogenic Compounds Evaluated in NSCLC

Ramucirumab

Ramucirumab is an IGG1 humanized monoclonal antibody targeting the VEGFR-2 receptor. It is being evaluated in a number of disease sites including NSCLC. A phase II trial of ramucirumab in combination with carboplatin and paclitaxel was reported at the American Society of Clinical Oncology meeting in 2010 (Table 3) [41]. Data were reported on 31 patients with NSCLC (all histologies were allowed). There were 10 responses in 15 evaluable patients (RR 67 %). Detailed toxicity information was not available.

Table 3 Other intravenous anti-angiogenic compounds that have been evaluated in NSCLC

Trial	Design	Intervention	Number	ORR	PFS	OS	**QoL**
First-line therapy							
Ramucirumab							
Camidge [41]	Phase I	Cb+Pac+Ram	22 evaluable	10/15 (67 %)	5.7 m (95 % CI 5.62–5.75)	NR	NA
Doebele [42]	Randomized phase II	Cis/Cb+Pem+Ram 10 mg/kg → Pem+Ram Cis/Cb+Pem → Pem	69 71	49.3 % 38 %	7.2 m (HR 0.75, 90 % CI 0.55–1.03) 5.6 m	13.9 m (HR 0.83, 90 % CI 0.56–1.22) 10.4 m	NA
Aflibercept							
Chen [44]	Phase II	Cis+Pem+Aflib	42	26.3 %	5 m (95 % CI 4.3–7.1 m)	NR	Trial stopped early because 3 cases RPLS
Vadimezan							
McKeage [47]	Randomized phase II	Cb+Pac+Vad → Vad Cb+Pac	37 36	34.4 % 29 %	5.4 m (HR 0.86, 0.5–1.4) 4.4 m	14.0 m (HR 0.73, 0.39–1.38) 8.8 m	NA
ATTRACT-1 [48]	Phase III	Cb+Pac+Vad → Vad Cb+Pac+Plac → Plac	649 650	24.7 % 24.6 %	5.5 m (HR 1.04, 0.–91 1.19) 5.5 m	13.4 m (HR 1.01, 0.85–1.19) 12.7 m	EORTC QLQ30 – no differences observed
Fosbretabulin							
FALCON [110]	Randomized phase II	Carb+Pac+Bev+Fos Carb+Pac+Bev	31 29	55 % 38 %	8.6 m (HR 0.89, 0.46–1.73) 8.8 m	–	NA
Second-line therapy							
VITAL [45]	Phase III	Doc+Aflib Doc+Plac	456 457	23.3 % 8.9 %	5.2 m (HR 0.82, 0.72–0.94) 4.1 m	10.1 m (HR 1.01, 0.87–1.17) 10.4 m	LCSS – no differences observed

Cb carboplatin, *Pac* paclitaxel, *Ram* ramucirumab, *Cis* cisplatin, *Pem* pemetrexed, *Doc* docetaxel, *Aflib* aflibercept, *RPLS* reversible posterior leukoencephalopathy syndrome, *Vad* vadimezan, *Plac* placebo, *Fos* fosbretabulin

Doebele et al, reported the results of a randomized phase II trial of cisplatin or carboplatin in combination with pemetrexed, with or without ramucirumab for four to six cycles [42]. Pemetrexed was continued as maintenance therapy either alone or with ramucirumab. A total of 140 patients were randomized. The addition of ramucirumab appeared to improve the efficacy of a platinum agent plus pemetrexed. Higher response rates were observed (49.3 % vs. 38 %). There were trends towards improved PFS (HR 0.75, 90 %CI 0.55–1.03) and OS (HR 0.83, 90 %CI 0.56–1.22), although these did not achieve statistical significance. Patients randomized to ramucirumab experienced more grade 3 neutropenia, anemia, thrombocytopenia, nausea, fatigue, hypertension, and back pain.

At this point ramucirumab remains an investigational agent with some promise. There are ongoing randomized phase II trials in both first and second-line therapy for NSCLC.

Aflibercept

Aflibercept is a human fusion protein designed to block biding of VEGF-A and VEGF-B, as well as human placental growth factor (commonly referred to as VEGF-trap). It has evidence of minor single agent activity in heavily pretreated patients [43]. A phase II trial of first-line therapy with aflibercept in combination with cisplatin and pemetrexed showed modest activity [44]. The RR was 26.3 % with median PFS of 5 months. However, the trial was stopped early because of three cases of reversible posterior leukoencephalopahy syndrome (RPLS). A randomized phase III trial of docetaxel plus or minus aflibercept as second-line therapy, showed some evidence of activity for the addition of aflibercept [45]. The addition of aflibercept to docetaxel improved RR (23.3 % vs. 8.9 %, $p<0.001$) and PFS (HR 0.82, 95 %CI 0.72–0.94). However, the study failed to achieve its primary outcome of improved overall survival. In addition there were no improvements in quality of life as measured by the Lung Cancer Symptom Scale (LCSS). There are multiple ongoing trials of aflibercept in other cancers. However, data to date do not support the use of aflibercept in NSCLC.

Vadimezan

Vadimezan is another VEGF-Trap compound [46]. Activity was observed in early phase clinical trials. A randomized phase II trial of carboplatin and paclitaxel plus/or minus vadimezan suggested that vadimezan may improve the effectiveness of platinum-based chemotherapy [47]. However, a phase III trial of the same regimen failed to improve on treatment outcomes [48]. There were no significant differences in response rates, PFS and overall survival. Additionally, quality of life between the two groups was similar. Therefore, vadimezan does not improve the therapeutic options for patients with advanced NSCLC.

Small Molecule Oral Anti-Angiogenic Compounds in the First-Line Therapy of NSCLC

There are multiple small molecule anti-angiogenic compounds that have been evaluated in combination with platinum-based chemotherapy, in the first-line therapy for NSCLC (Table 4). These agents inhibit one or more angiogenic pathways (VEGFR, PDGFR and/or FGFR), in addition to other off target receptors (Table 1). To date, despite promising preliminary data, none of these compounds have improved the therapeutic efficacy of platinum-based chemotherapy alone. Drug related toxicities of some agents preclude the administration of full doses when combined with chemotherapy. Incremental toxicity was seen in all the trials and in some cases appears to increase the risk of death. As such there are no data to support the addition of an oral anti-angiogenic agent to platinum-based chemotherapy.

Sorafenib

Sorafenib is a multitargeted TKI active against VEGFR, PDGFR, Raf, c-Kit and FLT-3 [49]. Preliminary data from heavily pretreated NSCLC patients showed disease stabilization in 59 % of patients [50]. As a result, sorafenib underwent further development in NSCLC. A phase I/II trial of sorafenib in combination with standard doses of carboplatin and paclitaxel demonstrated sorafenib could be administered in full doses with chemotherapy [51]. Subsequently, two phase III trials were conducted [52, 53]. The ESCAPE trial randomized 926 patients to carboplatin and paclitaxel plus sorafenib or placebo [53]. Patients were all good performance status (ECOG 0-1) and all NSCLC histologies were included in the trial. The trial was stopped early following an interim analysis that met criteria for futility. At the time of the final analysis, no differences were observed in OS between the two groups (median OS 10.7 m vs. 10.3 m, HR 1.15, 95 %CI 0.94–1.41). Additionally, there were no differences observed in the secondary outcomes (RR or PFS) and quality of life was not assessed in the trial. Interestingly a planned analysis according to histology demonstrated that patients with squamous cancer randomized to sorafenib had worse OS than patients in the placebo group (8.9 m vs. 13.6 m, HR 1.85, 95 %CI 1.22–2.81). Patients randomized to sorafenib experienced more thrombocytopenia, rash / desquamation, hand-foot reaction, hypertension and pruritis than in the placebo group. However, patients with squamous cancer did not experience incrementally worse toxicity.

A similarly designed trial (NEXUS), evaluated the addition of sorafenib or placebo, to cisplatin and gemcitabine in 772 patients [52]. Recruitment of patients with squamous cancer was halted following analysis of the ESCAPE trial. Data have been reported on the non-squamous histology population. No improvement in OS was observed for patients randomized to sorafenib (12.4 m vs. 12.5 m, HR 0.98, 95 %CI 0.83–1.16). A similar toxicity profile was observed to that seen in the ESCAPE trial. In summary there is no evidence to support the addition of sorafenib to first-line platinum-based chemotherapy.

Table 4 Summary of trials of oral anti-angiogenic agents in combination with platinum-based chemotherapy as first-line therapy for NSCLC

Trial	Design	Intervention	Number	ORR	PFS	OS	QoL
Sorafenib							
ESCAPE [53]	Phase III	Cb+Pac+Soraf Cb+Pac+Plac	464 462	27.4 % 24 %	4.6 m (HR 0.99, 0.84–1.16) 5.4 m	10.7 m (HR 1.15, 0.94–1.41) 10.3 m	NA Trial stopped early for futility
NEXUS [52]	Phase III	Cis+Gem+Soraf Cis+Gem+Plac	385 387	28 % 26 %	6.0 m (HR 0.83, 0.71–0.97) 5.5 m	12.4 m (HR 0.98, 0.83–1.16) 12.5 m	NA
Cediranib							
NCIC IND 171 [55]	Phase I	Cb+Pac+Ced	20	45 % (23–68 %)	7.6 m		NA
BR24 [56]	Randomized phase II	Cb+Pac+Ced 30 mg Cb+Pac+Plac	126 125	38 % 16 %	5.6 m (HR 0.77, 0.56–1.08) 5.0 m	HR 0.78 (0.57–1.06)	EORTC QLQ30 and LCS13 – not reported
BR29 [57]	Phase III	Cb+Pac+Ced 20 mg Cb+Pac+Plac	153 153	52 % 34 %	5.5 m (HR 0.91, 0.71–1.18) 5.5 m	12.2 m (HR 0.94, 0.69–1.30) 12.1 m	EORTC QLQ30 and LCS13 – not reported
NCCTG528 [58]	Randomized phase II	Cb+Gem+Ced 30 mg Cb+Gem+Plac	60 31	19 % 20 %	6.3 m (HR 0.69, 0.43–1.09) 4.5 m	12 m (HR 0.66, 0.41–1.08) 9.9 m	NA
Motesanib							
NCT00369070 [60]	Randomized phase II	Cb+Pac+Mot 125 mg daily Cb+Pac+Mot 75 mg bid Cb+Pac+Bev 15 mg/kg	61 62 63	30 % 23 % 37 %	7.7 m (HR 1.14, 0.73–1.76) 5.8 m (HR 1.22, 0.80–1.85) 8.3 m	14.0 m (HR 1.05, 0.67–1.63) 12.8 m (HR 1.18, 0.76–1.83) 14.0 m	NA
MONET-1 [61]	Phase III	Cb+Pac+Mot Cb+Pac+Plac	541 549	40 % 26 %	5.6 m (HR 0.79, 0.68–0.90) 5.4 m	13 m (HR 0.90, 0.78–1.04) 11 m	NA

(continued)

Table 4 (continued)

Trial	Design	Intervention	Number	ORR	PFS	OS	QoL
Vandetanib							
Heymach [64]	Randomized phase II	Cb+Pac+Vandet Vandet Cb+Pac	56 73 52	32 % 7 % 25 %	24w (HR 0.76, p=0.098) 11.5w (HR 1.26, p=0.86) 23w	10.2 m (HR 1.15, p=0.738) 10.2 m (HR 1.09, p=0.651) 12.6 m	NA
PrE0501 [63]	Randomized phase II	Cb+Doc+Vandet → Vandet Plac	162 total		4.5 m (p=0.07) 4.2 m	9.8 m (p=0.68) 9.4 m	NA
Pazopanib							
Scagliotti [66]	Randomized phase II	Pem+Pazop Cis+Pem	71 35	23 % 34 %	25w (HR 0.75, 0.43–1.28) 22.9w	HR 1.22 (0.64–2.33)	Trial stopped early because of increased treatment related deaths
Axitanib							
Belani [68]	Randomized phase II	Cis+Pem+Axit D1–21 Cis+Pem+Axit D2–19 Cis+Pem	55 58 57	45.5 % 39.7 % 26.3 %	8.0 m (HR 0.89, 0.56–1.4) 7.9 m (HR 1.02, 0.64–1.62) 7.1 m	16.6 m (HR 1.08, 0.66–1.760) 14.7 m (HR 1.39, 0.87–2.22) 15.9 m	NA

Cb carboplatin, *Pac* paclitaxel, *Soraf* sorafafenib, *Cis* cisplatin, *Gem* gemcitabine, *Ced* cediranib, *Mot* motesanib, *Bev* bevacizumab, *Vandet* vandetanib, *Do* decetaxel, *Pazop* pazopanib, *Pem* pemetrexed, *Axit* axitanib

Cediranib

Cediranib is an oral TKI with activity against VEGFR, PDGFR and c-Kit [54]. The recommended phase I dose of cediranib was 45 mg when combined with chemotherapy [55]. Several randomized trials have evaluated cediranib in combination with either carboplatin and paclitaxel [56, 57], or cisplatin and gemcitabine [58]. The NCIC Clinical Trials Group (CTG) BR24 trial evaluated an initial dose of cediranib of 45 mg daily or placebo, in combination with carboplatin and paclitaxel [56]. Excess toxicity was observed in the early phase of the trial necessitating a reduction of the cediranib dose to 30 mg daily. Despite this, there was an increase in hypertension, diarrhea, anorexia, fatigue, stomatitis, dyspnea and sensory peripheral neuropathy. Furthermore, there was an imbalance in treatment related deaths (10 vs. 2). However, it did appear as though the addition of cediranib improved RR (38 % vs. 16 %) and PFS (5.6 m vs. 5.0 m, HR 0.77, 95 %CI 0.56–1.08). A redesigned trial (NCIC BR29) utilizing a reduced dose of cediranib (20 mg daily) was commenced [57]. Despite the lower dose of cediranib, there was still an increase in toxicity for patients randomized to chemotherapy plus cediranib. Unfortunately, no differences in PFS or OS were observed in patients randomized to cediranib at the lower dose.

One further randomized phase II trial evaluated the addition of cediranib 30 mg daily to cisplatin and gemcitabine [58]. The toxicity profile was consistent with that observed in previous studies. No significant differences in PFS or OS were observed. The available data does not support the use of cediranib in NSCLC. While there was some evidence of increased efficacy from the addition of cediranib to chemotherapy, the increased toxicity and small risk of death necessitated dose reductions to the point where there was no benefit beyond standard chemotherapy. The lack of predictive biomarkers does not justify further development of this agent in NSCLC.

Motesanib

Motesanib is an oral TKI with activity against VEGFR, PDGFR, c-Kit and RET [59]. Development of motesanib in NSCLC has been limited to first-line therapy in combination with chemotherapy. An initial randomized phase II trial evaluated two different doses of motesanib (75 mg BID and 125 mg daily) combined with carboplatin and paclitaxel, compared with carboplatin, paclitaxel plus bevacizumab [60]. Similar activity was observed for the combination of motesanib 125 mg daily, plus carboplatin and paclitaxel and carboplatin, paclitaxel and bevacizumab.

The phase III MONET-1 trial randomized NSCLC patients to carboplatin, paclitaxel with or without motesanib [61]. All histologies were initially included. However, an interim analysis demonstrated an increased risk of death and gross hemoptysis for patients with squamous histology. Further recruitment was limited to patients with non-squamous histology. There were 1090 patients with non-squamous histology randomized on trial. Patients randomized to motesanib plus

chemotherapy had higher RR (40 % vs. 26 %) and longer PFS (5.6 m vs. 5.4 m, HR 0.79, 95 %CI 0.68–0.90) compared with chemotherapy alone. Treatment with motesanib did not significantly improve OS (13 m vs. 11 m, HR 0.90, 95 %CI 0.78–1.04). There was a higher incidence of neutropenia (22 % vs. 15 %), diarrhea (9 % vs. 1 %) and hypertension (7 % vs. 1 %) among patients receiving motesanib. Biomarker analysis was performed to evaluate Placental Growth Factor (PLGF). Neither baseline PLGF, nor change in PLGF from baseline to week four were associated with improved survival for patients randomized to motesanib. This agent is not being further developed in NSCLC.

Vandetanib

Vandetanib is a multitargeted TKI that inhibits VEGFR, EGFR and RET [62]. Dual inhibition of both VEGFR and EGFR offers the potential to inhibit two important molecular pathways in NSCLC. To date though, the addition of vandetanib to first-line therapy has not improved the therapeutic ratio.

Two randomized phase II trials have evaluated vandetanib in first-line therapy [63, 64]. Heymach et al, randomized patients to carboplatin, paclitaxel and vandetanib, carboplatin, paclitaxel alone, or single agent vandetanib (Table 4) [64]. Recruitment to single agent vandetanib was discontinued following an interim analysis showing lower efficacy. Median PFS was similar between carboplatin and paclitaxel alone or in combination with vandetanib (HR 0.76, 95 %CI 0.51–1.14) and there was no improvement in OS (HR 1.15, 95 %CI 0.75–1.77). A second trial evaluated maintenance vandetanib or placebo following initial therapy with carboplatin, docetaxel and vandetanib [63]. There was no significant improvement in PFS (4.5 vs. 4.2 months; $p = 0.07$) and OS (9.8 vs. 9.4 months; $p = 0.68$) with vandetanib versus placebo. Common toxicities associated with vandetanib include rash, diarrhea and hypertension. Data do not support the addition of vandetanib to first-line treatment options.

Pazopanib

Pazopanib is another multi-targeted TKI with activity against VEGFR, PDGFR and FGFR [65]. There is limited evaluation of pazopanib in the metastatic setting. A randomized phase II trial compared pemetrexed plus cisplatin with pemetrexed plus pazopanib [66]. The trial was halted after 106 of 150 patients were entered, because of an increased risk of death in the pazopanib group. There was a higher frequency of grade 3 and 4 neutropenia observed among patients randomized to 800 mg daily of pazopanib, necessitating a dose reduction to 600 mg daily. Despite this the trial was discontinued. No significant differences were observed in any of the trial outcomes. PFS favoured patients randomized to pazopanib, although RR

and OS favoured cisplatin and pemetrexed. Given the observed toxicity from pazopanib, it should not be used in combination with chemotherapy in the treatment of NSCLC.

Axitanib

Axitanib is a TKI inhibiting VEGFR, PDGFR and c-Kit with modest activity as a single agent [67]. A randomized phase II trial evaluated cisplatin and pemetrexed alone, or in combination with two dose schedules of axitinib [68]. Higher RRs were seen in patients in both axitanib arms, although there were no significant differences in PFS or OS. This agent remains investigational as a treatment for NSCLC.

Trials of Oral Anti-Angiogenic Compounds after Failure of First-Line Therapy for NSCLC

At least six oral anti-angiogenic agents, either alone, or in combination with other agents, have been evaluated in the treatment of NSCLC after failure of first-line therapy (Table 5). Many of these agents have some evidence of activity in NSCLC. Improvements in PFS have been observed, but none of the agents have demonstrated improved OS compared with existing standards of care.

Sorafenib

Sorafenib has been extensively evaluated in NSCLC patients progressing after platinum-based chemotherapy. A novel randomized discontinuation design was used to evaluate sorafenib in patients who had at least two prior chemotherapy treatments and an EGFR TKI [69]. All patients (n = 299) received 2 months of sorafenib 400 mg twice daily. Responding patients continued sorafenib, while patients who progressed came off treatment. Those patients with stable disease were randomized to continue or discontinue their sorafenib (n = 105). Due to an error in the randomization schedule, eight patients randomized to sorafenib initially received placebo and 12 patients on the placebo arm received sorafenib, which confounds interpretation of the data. There was a significant improvement in PFS for patients randomized to sorafenib (3.3 m vs. 2.0 m, HR 0.51, 95 %CI 0.30–0.87). Differences were also observed in OS (13.7 m vs. 9.0 m, HR 0.67, 95 %CI 0.40–1.11), although these were not statistically significant. Further investigation of sorafenib in this situation appears warranted.

Additional trials have evaluated the addition of sorafenib to either erlotinib [70], or pemetrexed [71]. The Lun 160 trial randomized patients who had failed one or

two lines of chemotherapy, to erlotinib plus sorafenib, or erlotinib plus placebo (2:1 randomization) [70]. There was no difference in RR between the two arms (8 % vs. 11 %). PFS favoured the combination of erlotinib and sorafenib but the difference was not statistically significant (HR 0.86, 95 %CI 0.60–1.22). Analysis of biomarkers for EGFR and KRAS were inconclusive, however data raised the potential for benefit from the combination of erlotinib and sorafenib in patients with *EGFR* WT, or *EGFR* FISH negative tumors. E2501 randomized patients eligible for second-line chemotherapy, to pemetrexed alone or in combination with sorafenib [71]. No differences were observed in PFS or OS, therefore this combination does not appear to warrant further evaluation.

Sunitinib

Sunitinib has also been evaluated in combination with both chemotherapy and erlotinib after failure of first-line platinum-based chemotherapy (Table 5). CALGB 30704 randomized patients to pemetrexed alone, sunitinib alone, or the combination of pemetrexed plus sunitinib as second-line therapy for advanced NSCLC [72]. One hundred and thirty patients were randomized. A higher RR to treatment was observed in patients receiving the combination of pemetrexed plus sunitinib, although this difference was not statistically significant. However, both PFS and OS favored single agent pemetrexed. In regards OS, patients randomized to the combination of pemetrexed plus sunitinib had significantly worse survival (HR 2.0, 95 %CI 1.2–3.2). Therefore, current data do not support the addition of sunitinib to second-line chemotherapy.

Blumenschein et al. [73], reported on the safety and pharmacokinetics from a lead-in cohort of a randomized phase II trial of the combination of erlotinib 150 mg daily with or without sunitinib 37.5 mg daily continuously. Thirty patients were enrolled. Sunitinib administration did not affect the pharmacokinetic profile of erlotinib, but did reduce the exposure of sunitinib. Two subsequent randomized trials evaluated the addition of sunitinib to erlotinib [74, 75]. Groen et al., randomized 132 patients to erlotinib plus sunitinib versus erlotinib plus placebo [74]. There were no differences observed in either PFS or OS. A larger phase III trial of the same design demonstrated a significant improvement in PFS for patients randomized to erlotinib plus sunitinib (median PFS 3.6 m vs. 2.0 m, HR 0.81, 95 %CI 0.69–0.94) [76]. However, these differences did not translate into ay improvements in OS (median OS 9.0 m vs. 8.5 m, HR 0.92, 95 %CI 0.80–1.07). Additionally there were no differences observed in quality of life as measured by the EQ-5D. Common grade 3/4 toxicities observed in these studies include fatigue and asthenia. In studies combining sunitinib with erlotinib additional toxicities included rash, diarrhea, dyspnea, thrombocytopenia, and neutropenia. Given the increase in toxicity together with a lack of improvement in survival, data suggest that sunitinib should not be used in the management of NSCLC following progression of first-line therapy.

Table 5 Summary of trials of oral anti-angiogenic agents after failure of first-line therapy for NSCLC

Trial	Design	Intervention	Number	ORR	PFS	OS	QoL
Sorafenib							
NCT00922584 [50]	Phase II	Soraf	52	0 %	2.7 m	6.7 m	NA
E2501 [69]	Randomized phase II	Soraf induction → Soraf Plac	299 enrol 53 52	3 %	3.3 m (HR 0.51, 0.30–0.87) 2.0 m	13.7 m (HR 0.67, 0.40–1.11) 9.0 m	NA
Lun160 [70]	Randomized phase II	Erl+Soraf Erl+Plac	112 56	8 % 11 %	3.38 m (HR 0.86, 0.60–1.22) 1.94 m	7.62 m (HR 0.89, 0.59–1.34) 7.23 m	NA
NCCTG626 [71]	Randomized phase II	Pem+Soraf Pem	49 51		3.4 m (p=0.22) 4.1 m	9.4 m (p=0.49) 9.7 m	NA
Sunitinib							
CALGB30704 [72]	Randomized phase II	Pem+Sun Sun Pem	42 47 41	22 % 17 % 14 %	3.7 m (HR 1.3, 0.9–2.1) 3.3 m (HR 1.4, 0.9–2.2) 4.9 m (p=0.3)	6.7 m (HR 2.0, 1.2–3.2) 8.0 m (HR 1.4, 0.9–2.3) 10.5 m	NA
Blumenschein [73]	Phase II	Erl + Sun Erl	13 17	NR	NR	NR	no effect on ERL PK
Groen [74]	Randomized phase II	Erl+Sun Erl+Plac	132 total	NR	12.3w (HR 0.95, 0.60–1.5) 8.5w	8.2 m (HR 1.09, 0.72–1.6) 7.6 m	NA
Scagliotti [76]	Phase III	Erl+Sun Erl+Plac	480 480	10.6 % 6.9 %	3.6 m (HR 0.81, 0.69–0.94) 2.0 m	9.0 m (HR 0.92, 0.80–0.1.07) 8.5 m	EQ–5D – no difference between the two arms
Vandetanib							
ZEAL [77]	Phase III	Pem+Vandet Pem+Plac	256 278	19 % 8 %	17.6w (HR 0.86, 0.69–1.06) 11.9w	10.5 m (HR 0.86, 0.65–1.13) 9.2 m	LCSS – longer time to deterioration in total LCSS score for vandet

(continued)

Table 5 (continued)

Trial	Design	Intervention	Number	ORR	PFS	OS	QoL
ZODIAC [78]	Phase III	Doc+Vandet Doc+Plac	694 697	17 % 10 %	4.0 m (HR 0.79, 0.70–0.90) 3.2 m	10.6 m (HR 0.91, 0.78–1.07) 10.0 m	FACT–L LCS. Delayed time to symptom progression in Vandet
ZEST [80]	Phase III	Vandet Erl	623 617	12 % 12 %	2.6 m (HR 0.98, 0.87–1.10) 2.0 m	6.9 m (HR 1.01, 0.89–1.16) 7.8 m	EORTC QLQ30 – no difference in time to deterioration in symptoms
ZEPHYR [79]	Phase III	Vandet Plac	617 307	2.6 % 0.7 %	1.9 m (HR 0.63, 0.54–0.74) 1.8 m	8.5 m (HR 0.95, 0.81–1.11) 7.8 m	FACT–L LCS. No significant difference between groups
Nintedanib							
Reck [83]	Randomized phase II	Ninted 150 mg bid Ninted 250 mg bid	37 36	0 % 2.8 %	53d 48d	20.6w 29.7w (HR 0.69, p = 0.21)	
Lume-Lung1 [86]	Phase III	Doc+Ninted Doc+Plac	652 659	4.7 % 3.6 %	3.4 m (HR 0.79, 0.68–0.92) 2.7 m	10.1 m (HR 0.94, 0.83–1.05) 9.1 m	NA
Lume-Lung2 [85]	Phase III	Pem+Ninted Pem+Plac	353 360	9.1 % 8.3 %	4.4 m (HR 0.83, 0.70–0.99) 3.6 m	12.2 m (HR 1.03, 0.85–1.24) 12.7 m	NA
Cediranib							
Gadgeel [87]	Phase II	Pem+Ced	33	16 %	–	–	NA
Linifanib							
Tan [89]	Phase II	Linif	139	1.4 %	3.6 m	9.0 m	NA

Soraf sorafenib, *Plac* placebo, *Erl* erlotinib, *Pem* pemetrexed, *Sun* sunitinib, *PK* pharmacokinetics, *Vandet* vandetanib, *Ninted* nintedanib, *Doc* docetaxel, *Ced* cediranib, *Linif* linifanib

Vandetanib

Vandetanib has been evaluated in four randomized phase III trials following progression of first-line platinum-based chemotherapy, either in combination with pemetrexed [77], or docetaxel [78], or as a single agent [79, 80]. The ZEAL trial randomized 534 NSCLC patients to second-line therapy with pemetrexed plus placebo, or pemetrexed plus vandetanib 100 mg daily [77]. The primary outcome of the study, PFS, was not significantly prolonged among patients randomized to pemetrexed plus vandetanib (median PFS 17.6w vs. 11.9w, HR 0.86, 95 %CI 0.69–1.06). A higher RR was observed (19 % vs. 8 %, $p < 0.001$), but this did not translate into any improvements in OS for patients randomized to the combination arm (10.5 m vs. 9.2 m, HR 0.86, 95 %CI 0.65–1.13). Quality of life was measured using the Lung Cancer Symptom Scale (LCSS). Patients randomized to pemetrexed and vandetanib had a significantly longer time to deterioration of lung cancer symptoms than patients randomized to pemetrexed alone. A similarly designed trial (ZODIAC) randomized patients to docetaxel along or in combination with vandetanib [78]. There were 1391 patients randomized on study. A significant improvement in PFS was observed for patients randomized to docetaxel plus vandetanib (4.0 m vs. 3.2 m, HR 0.79, 95 %CI 0.70–0.90), but no significant differences were observed in OS (10.6 m vs. 10.0 m, HR 0.91, 95 %CI 0.78–1.07). Time to deterioration in symptoms significantly favored patients randomized to docetaxel plus vandetanib. While there is some evidence of increased activity for the addition of vandetanib to second-line chemotherapy, the lack of improvement in overall survival means that this agent has not been incorporated into treatment algorithms for advanced NSCLC.

Additional trials have evaluated vandetanib after prior chemotherapy. The ZEST trial compared vandetanib 300 mg daily with erlotinib 150 mg daily in patients who had received one or two prior chemotherapy treatments [80]. There were 1240 patients randomized. Vandetanib did not improve PFS in comparison to erlotinib (2.6 m vs. 2.0 m, HR 0.98, 95 %CI 0.87–1.10). Similarly there were no differences observed in OS (6.9 m vs. 7.8 m, HR 1.01, 95 %CI 0.89–1.16). There were also no difference in time to deterioration in symptoms between the two groups, as assessed by the EORTC-QLQ30 and LDS-13. While vandetanib demonstrated similar activity to erlotinib, the trial was designed to show superiority of vandetanib over erlotinib. Therefore, this is considered to be a negative trial.

The last phase III trial evaluating vandetanib, compared vandetanib versus placebo in patients who had received prior chemotherapy and an EGFR TKI (ZEPHYR) [81]. Nine hundred and twenty four patients were randomized to vandetanib 300 mg daily or placebo (2:1 schedule). The trial failed to demonstrate any improvement in OS (8.5 m vs. 7.8 m, HR 0.95, 95 %CI 0.81–1.11) and there were no differences observed in quality of life, as measured by the FACT-L. Therefore, none of the four trial of vandetanib after progression of platinum-doublet chemotherapy show any improvement in OS in comparison to standard treatment options. Given the observed toxicity of vandetanib, it has not been incorporated into the treatment algorithms for NSCLC.

Nintedanib

Nintedanib is an oral triple angiokinase inhibitor with activity against VEGFR, PDGFR, and FGFR, as well as members of the Src family and flt-3 [82]. A randomized phase II trial of two dose levels of nintedanib reported similar efficacy of single agent nintedanib 150 mg BID and 250 mg BID using a continuous dose schedule [83]. A subsequent phase I trial of nintedanib in combination with pemetrexed, reported that the recommended dose of nintedanib in combination with chemotherapy was 200 mg BID [84]. This schedule was used in two subsequent phase III trials evaluating nintedanib, or placebo in combination with either pemetrexed [85], or docetaxel [86].

The Lume-Lung 2 trial evaluated the addition of nintedanib or placebo to pemetrexed in patients with non-squamous histology [85]. This has been presented in abstract only. The trial was discontinued early following an interim analysis suggesting futility. At the time of the final analysis, there was no difference in RR (9.1 % vs. 8.3 %). However, there was a significant improvement in PFS observed (4.4 m vs. 3.6 m, HR 0.83, 95 %CI 0.70–0.99). This did not translate into any improvement in OS (12.2 m vs. 12.7, HR 1.03, 95 %CI 0.85–1.24). The Lume-Lung 1 trial evaluated nintedanib or placebo, plus docetaxel in patients with all NSCLC histologies [86]. The primary outcome was defined as PFS. OS for the entire study population was a secondary outcome. Prior to the database lock for OS, but after the PFS analysis was performed this was modified to include a pre-specified sequence for analysis for OS: first in adenocarcinoma histology who progressed within 9 months from start of initial first-line therapy; next in all adenocarcinoma histologies; and then in all NSCLC histologies. It is important to recognise that while this was done to validate subgroup analyses from the Lume-Lung 2 trial, it involves some comparisons that are no longer randomized. The trial demonstrated a significant improvement in PFS for the overall study population (3.4 m vs. 2.7 m, HR 0.85, 95 %CI 0.75–0.96). In the analysis of OS, patients randomized to docetaxel and nintedanib with adenocarcinoma and less than 9 months from start of first-line chemotherapy to study entry, demonstrated improved OS in comparison to patients randomized to docetaxel plus placebo (10.9 m vs. 7.9 m, 95 %CI 0.60–0.92). A significant improvement was also observed in all patients with adenocarcinoma histology (12.6 m vs. 10.3 m, HR 0.83, 95 %CI 0.70–0.93). However, no difference was observed in OS for the original study population involving all histologies (10.1 m vs. 9.1 m, HR 0.94, 95 %CI 0.83–1.05). Adverse events occurring more frequently in patients receiving nintedanib included diarrhea, increased liver enzymes (ALT and AST), nausea, vomiting and decreased appetite. Excess hypertension was not observed with this agent.

Nintedanib is the only oral anti-angiogenic agent evaluated in NSCLC patients following failure of first-line therapy to demonstrate any improvement in OS. However, these findings should be considered hypothesis generating at this time. There are plans to repeat this study with a population limited to adenocarcinoma histology in NSCLC patients who progressed within 9 months of starting first-line therapy.

Cedirinib

There are no randomized data of cediranib following progression of first-line platinum-based therapy. Preliminary results from a single arm phase II trial of pemetrexed plus cediranib was presented at the ASCO meeting in 2009 [87]. The observed RR was 16 % with an acceptable toxicity profile. However, the lack of a suitable control group makes interpretation of these data challenging. This agent is not undergoing further development in NSCLC

Linifanib

Linifanib is an oral anti-angiogenic agent active against VEGFR and PDGFR [88]. Only one phase II trial was identified in which 139 patients were treated with single agent linifanib after progression on prior chemotherapy treatments [89]. The observed activity was modest and this agent remains experimental at this time.

Anti-Angiogenic Therapy in SCLC

There have been a number of trials evaluating targeted therapies in SCLC. To date, trials have evaluated matrix metalloproteinases [90], inhibitors of c-myc with imatinib alone [91] or in combination with systemic therapy [92], BCL-2 antisense therapy [93], proteosome inhibition [94] and inhibition of insulin-like growth factor (IGF) [95]. All of these have been ineffective strategies that have failed to improve survival for patients with SCLC. One potential explanation is the complexity of molecular abnormalities occurring in SCLC [96, 97]. Inhibition of VEGF is a strategy that has been routinely incorporated into the therapy of NSCLC. Multiple anti-angiogenic compounds have been evaluated in SCLC, although to date not have been proven to improve survival of patients (Table 6).

Bevacizumab

Two single arm phase II trials have evaluated the addition of bevacizumab to either cisplatin and irinotecan [98], or cisplatin or etoposide [99]. Ready et al, included 68 patients in a phase II trial of cisplatin, irinotecan and bevacizumab [98]. The RR was 75 % and median PFS 7.0 months (95 %CI 6.4–8.4 m). The median OS was 11.6 months (95 %CI 10.5–15.1 m). Common toxicities of the combination include neutropenia, nausea, diarrhea, dehydration and fatigue. A second phase II trial evaluated cisplatin, etoposide and bevacizumab [99]. Efficacy was consistent with that

Table 6 Summary of trials of anti-angiogenic therapy in SCLC

Trial	Design	Intervention	Number	ORR	PFS	OS	QoL
Bevacizumab							
CALGB 30306 [98]	Phase II	Cis+Irino+Bev	68	75 %	7.0 m (95 %CI 6.4–8.4 m)	11.6 m (95 %CI 10.5–15.1 m)	NA
E3501 [99]	Phase II	Cis+Etop+Bev 15 mg/kg	65	63.5 %	4.7 m (95 %CI 4.3–5.5 m)	10.9 m (95 %CI 7.9–12.2 m)	NA
SALUTE [100]	Randomized phase II	Cis/Carb+Etop+Bev 15 mg/kg Cis/Carb+Etop+Plac	52 50	58 % 48 %	5.5 m (HR 0.53, 0.32–0.86) 4.4 m	9.4 m (HR 1.16, 0.66–2.04) 10.9 m	NA
Vandetanib							
NCICBR20 [101]	Randomized phase II	Cis+Etop → Vandet Plac	53 54		2.7 m (HR 1.01, 0.75–1.36) 2.8 m	10.6 m (HR 1.43, 1.0–2.05) 11.9 m	EORTC QLQ30+LCS13. QoL similar between the two arms
Thalidomide							
IFCT 00-01 [102]	Phase III	Epi+Cis+Etop+Cyc x 2 → Epi+Cis+Etop+Cyc+Thal Epi+Cis+Etop+Cyc+Plac	119 49 43	87 % 84 %	6.6 m (HR 0.74, 0.49–1.12) 6.4 m	11.7 m (HR 0.74, 0.49–1.12) 8.7 m	NA
Lee [103]	Phase III	Cb+Etop+Thal Cb+Etop+Plac	365 359	NR	No difference	10.5 m (HR 1.10, 0.94–1.29) 10.2 m	NA
Ellis [104]	Phase I	Cis+Etop+Pom	22	31.8 %		49.6w (95 %CI 28–63.9w)	NA

Sunitinib							
CALGB 30504 [106]	Randomized phase II	Cis/Cb+Etop→ Sunit Plac	138 44 41		3.8 m (HR 1.54, 90 %CI 1.03–2.3) 2.3 m	8.8 m (HR 1.1, 90 %CI 0.71–1.7) 6.7 m	NA
Han [105]	Phase II second line	Sunit	25	9 %	1.4 m (95 %CI 0.7–2.1 m)	5.6 m (95 % CI 3.2–8.0 m)	NA
Sorafenib							
Sharma [108]	Phase II	Cis+Etop+Soraf	17	67 %	–	7.4 m	Stopped because of toxicity concerns
Sharma [108]	Phase II	Cis+Etop+Soraf	17	67 %	–	7.4 m	Stopped because of toxicity concerns
S0435 [107]	Phase II second line	Soraf	89	4 %	2 m	7 m	NA

Cis cisplatin, *Irino* irinotecan, *Bev* bevacizumab, *Etop* etoposide, *Cb* carboplatin, *Vandet* vandetanib, *Plac* placebo, *Epi* epirubicin, *Cyc* cyclophosphamide, *Thal* thalidomide, *Pom* pomalidomide, *Sunit* sunitinib, *Soraf* sorafenib

expected from standard chemotherapy. The observed RR was 63.5 %, with a median PFS of 4.7 months (95 %CI 4.3–5.5 m). Median OS was 10.9 months (95 %CI 7.9–12.2 m). Common toxicities included neutropenia, thrombocytopenia, fatigue and weakness. Toxicities attributed to bevacizumab included hypertension, epistaxis, pulmonary and abdominal hemorrhage.

Bevacizumab has also been evaluated in a randomized phase II trial with cisplatin/carboplatin plus etoposide [100]. One hundred and two patients were randomized. There was no significant improvement in RR (58 % vs. 48 %). Progression free survival significantly favored the combination of cisplatin/carboplatin, etoposide plus bevacizumab (5.5 m vs. 4.4 m, HR 0.53, 95 %CI 0.32–0.86). This did not translate into any improvement in OS, however (9.4 m vs. 10.9 m, HR 1.16, 95 %CI 0.66–2.04). Therefore, available data do not support the addition of bevacizumab to standard chemotherapy in SCLC.

Vandetanib

One randomized trial evaluated vandetanib as maintenance therapy in SCLC [101]. The NCIC CTG BR20 trial included patients with both limited stage (LS) and extensive stage (ES) SCLC, with no evidence of disease progression following standard chemotherapy. One hundred and seven patients were randomized to vandetanib or placebo. There was no improvement in PFS (2.7 m vs. 2.8 m, HR 1.01, 95 %CI 0.75–1.36) and a trend to worse OS (10.6 m vs. 11.9 m, HR 1.43, 95 %CI 1.0–2.05). Toxicities were consistent with those previously observed from vandetanib. Therefore, there is no evidence to support the use of vandetanib in SCLC.

Thalidomide and Other Immunomodulatory Agents

Thalidomide is an anti-angiogenic agent suppressing FGF and VEGF. The IFCT-00-01 trial evaluated thalidomide in patients with ES SCLC who had evidence of tumor response following two cycle of induction therapy [102]. Patients were randomized to four additional cycles of chemotherapy with thalidomide 400 mg daily or placebo. Initially 119 patients were registered. The RR was 81.5 % and 92 patients were randomized to thalidomide or placebo. There was no significant difference observed in PFS (6.6 m vs. 6.4 m, HR 0.74, 95 %CI 0.49–1.12). Median OS was greater among patients receiving thalidomide, although this difference was not statistically significant (11.7 m vs. 8.7 m, HR 0.74, 955CI 0.49–1.12). Patients on the thalidomide arm experienced more neuropathy, but other toxicities did not differ. A second randomized trial evaluated thalidomide or placebo, in combination with carboplatin and etoposide [103]. Seven hundred and twenty four patients were randomized and no differences were observed in overall survival. As a result, thalidomide does not appear to be an active agent when added to standard chemotherapy.

A phase I trial of pomalidomide, an immunomodulatory agent, added to cisplatin and etoposide was conducted by Ellis et al. [104] Overlapping hematological toxicities did not allow pomalidomide to be administered at its recommended single agent dose. The observed response rates and overall survival were not sufficiently active to warrant further development.

Sunitinib

Two trials have evaluated sunitinib in SCLC. A phase II trial of sunitinib as second-line therapy showed some evidence of activity [105]. The RR was modest (9 %) and the median OS was 5.6 months (95 %CI 3.2–8.0 m). Only 25 patients were included and it is difficult to make any clear recommendations from this. However, there was some evidence of benefit from sunitinib as maintenance therapy. The CALGB 30504 initially planned to evaluate the combination of a platinum and etoposide in combination with sunitinib [106]. However, the observed toxicity was not tolerable. The trial was modified to evaluate maintenance sunitinib or placebo following initial therapy with cisplatin/carboplatin and etoposide. There was a significant improvement in PFS (3.8 m vs. 2.3 m, HR 1.54, 90 %CI 1.03–2.3). Overall survival also favored maintenance sunitinib although this difference was not statistically significant (8.8 m vs. 6.7 m, HR 1.1, 90 %CI 0.71–1.7). These results were of sufficient interest to warrant a phase III trial of the same design. At present though, sunitinib should be considered an investigational agent in SCLC.

Sorafenib

Several single agent phase II trials of sorafenib have been conducted [107, 108]. Results are summarized in Table 6. The RR and OS observed from the combination of carboplatin or cisplatin plus etoposide in combination with sorafenib does not appear greater than that expected from chemotherapy alone [108]. These trials included only a small number of patients, but the results do not appear to justify larger scale trials of these combinations. A second-line trial of sorafenib also showed modest activity [107]. Available data do not provide a strong rationale for further evaluation of sorafenib in SCLC.

Other Anti-Angiogenic Compounds under Evaluation for Lung Cancer

A number of other anti-angiogenic agents have been evaluated in lung cancer (Table 7). In general these agents are in early phase development in trial involving multiple cancer types. Activity has been observed in NSCLC patients providing a rationale for further evaluation. These agents have been primarily evaluated in

Table 7 Summary of other anti-angiogenic agents under development in lung cancer

Agent	Mechanism of action	Stage of development	
MGCD265	MET, VEGFR, Ron, Tie-2	Phase I multiple cancers in combination with Erl [111] Phase I multiple cancers in combination with Doc [112]	One response seen in three NSCLC patients Two responses seen in nine NSCLC patients
BMS690514	EGFR, Her2, VEGFR	Phase I in multiple cancers with expansion in NSCLC [113] Phase I/II in NSCLC patients [114] Phase I multiple cancers in combination with Carb+Pac [115]	One response observed in NSCLC Two responses in 60 patients Five responses observed in eight NSCLC patients
Brivanib	FGFR, VEGFR	Randomized discontinuation design in multiple advanced solid tumors [116]	Insufficient activity in NSCLC to warrant further development
Pazopanib	VEGFR, PDGFR, FGFR	Phase II neoadjuvant study in stage I/II NSCLC [117] Randomized trial of pazopanib in resected NSCLC [109]	Tumor reduction in 30 of 35 patients, three responses Trial did not proceed to phase III as unable to administer planned treatment

Erl erlotinib, *Doc* docetaxel, *Carb* carboplatin, *Pac* paclitaxel

heavily pre-treated patients. The one exception is pazopanib. A planned randomized trial of adjuvant therapy with pazopanib did not proceed beyond a phase II component because of inability to administer the planned dose of treatment [109]. Further development of this agent seems unlikely.

Conclusions

Angiogenesis is felt to be a key factor in tumor growth and development and therefore an important therapeutic target in cancer. There have been a large number of trials evaluating multiple anti-angiogenic compounds in lung cancer, but the majority of results have been disappointing. Unfortunately the majority of these agents have not improved the results of treatment in comparison to existing standard treatments for lung cancer. Interestingly, there appears to be some differential effect of anti-angiogenic therapy according to histological subtype. Several agents, including sorafenib and motesanib, have demonstrated worse survival for patients with squamous histology and toxicity concerns excluded patients with squamous cancers from therapy with bevacizumab. In addition, anti-angiogenic therapies add incremental and at times, overlapping toxicities to existing lung cancer treatments.

In some cases, such as cediranib, these additional toxicities requires dose reduction to a point where the drug is no longer beneficial.

Bevacizumab remains the only approved anti-angiogenic agent in the treatment of lung cancer. Bevacizumab therapy is limited to patients with good performance status with non-squamous NSCLC, absence of brain metastases, and no major bleeding or thrombotic disorders. Meta-analyses of trials of bevacizumab in combination with platinum-based chemotherapy show a modest improvement in overall survival, but there are incremental toxicity concerns. Attempts to date, to identify additional subgroups of patients who derive greater benefit from bevacizumab have not been successful. More recently, non bevacizumab-based treatment strategies have emerged. Data suggest that cisplatin or carboplatin plus pemetrexed followed by maintenance pemetrexed until disease progression, provides an alternative to bevacizumab-based therapy.

Overall, the results of trials evaluating oral anti-angiogenic agents have been disappointing. The majority of agents evaluated in phase III trials have not shown significant improvements in survival for patients with lung cancer. The challenge is understanding whether these agents are ineffective, or whether the effects in a particular subgroup are lost among a larger, unselected population of patients. This may be the case for nintedanib, where no improvement was observed in OS in two trials, although a subgroup analysis of patients with adenocarcinoma and shorter time until disease progression suggested improved survival. There is a need to additional translational studies to answer these issues.

The data in SCLC is disappointing as well. In general, targeted therapies have not proven beneficial in SCLC. Available data has not demonstrated a benefit, although there is some promise for sunitinib as maintenance therapy after initial platinum and etoposide treatment. Further research is required to identify which, if any SCLC patients, might benefit from anti-angiogenic based therapy.

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Target Therapy in Lung Cancer

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Introduction

Lung cancer is an extremely heterogeneous disease, with well over 50 different histological variants recognized under the fourth revision of the World Health Organization (WHO) typing system [1]. Because these variants have differing genetic and biological properties correct classification of lung cancer is necessary to assure that lung cancer patients receive optimum management. Due to the recent understanding that histologic typing and *EGFR* mutation status are important for target the therapy in lung adenocarcinoma patients [2] there was a great need for a new classification that addresses diagnostic issues and strategic management to allow for molecular testing in small biopsy and cytology specimens. For this reason and in order to address advances in lung cancer treatment an international multidisciplinary classification was proposed by the International Association for the Study of Lung Cancer (IASLC), American Thoracic Society (ATS), and European Respiratory Society (ERS) [3],

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further increasing the histological heterogeneity and improving the existing WHO-classification. Is now the beginning of personalized therapy era that is ideally finalized to treat each individual case of lung cancer in different way.

Oncogenic EGFR Mutation

The EGFR family of TKs referred to as the HER or ErbB family, consists of four members – EGFR (HER1/ErbB1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). These members regulate many physiological processes and are involved in the modulation of cell proliferation, apoptosis, cell motility and neovascularisation, thus being able to induce important mechanisms related to cancerogenesis [4, 5]. The EGFR tyrosine kinase works through the auto-activation of the receptor via its homo/heterodimerization and autophosphorylation on tyrosine-rich cytosolic domains after the binding of the ligand. This leads to the beginning of two main downstream intermediate pathways: the PIK3CA/AKT1/MTOR pathway and the RAS/RAF1/MAP2K1/MAPK1 kinases [6]. There is evidence that the activated EGFR can also mediate signals through the STAT transcription factors [7, 8]. Improper activation and over-expression of EGFR-TK results in increased cell proliferation, survival, invasion and metastasis. This has been implicated in the pathogenesis and progression of many malignancies as well as in the poor prognosis of patients [7, 9, 10]. In malignant cells, including NSCLC cells, the activity of the receptor may become dysregulated and no longer under the control of inherent inhibitory mechanisms [11]. Spontaneous EGFR mutations often are oncogenic; that is, they activate the EGFR-signalling pathway in the absence of ligand and promote cell proliferation, survival and anti-apoptotic signals. These signalling networks make EGFR-mutated cells dependent on a functional EGFR for their survival, rendering them addicted to the receptor. Inhibition of EGFR leads to up-regulation of pro-apoptotic molecules and finally results in cell death through the activation of the intrinsic mitochondrial apoptotic pathway [12, 13]. There are several described mutations in the EGFR gene. The two most common are: (1) short in-frame deletions around the LREA motif of exon 19 (~45–50 % of mutations); and (2) a point mutation (CTG to CGG) in exon 21 that results in substitution of leucine by arginine at codon 858, L858R (~45–50 % of mutations) [14, 15]. These mutations are more frequently found in NSCLC with an adenocarcinoma histology, tumors in women, East Asians and never smokers [14–16]. EGFR mutations in lung cancers constitute one of the major subsets among those molecular aberrations occurring in lung cancers. The incidence of EGFR mutations in tumors with non-small-cell histology ranges from ~15 % in Caucasians to ~50 % in East Asians [17]; 95 % of such mutations have been found in adenocarcinomas [18]. Patients bearing EGFR mutations have shown favourable clinical outcomes even with conventional chemotherapy suggesting that EGFR may serve as a predictive factor as well as a prognostic factor [19]. Over 50 % of patients diagnosed with NSCLC present with stage IIIB or IV disease is not amenable to curative treatment [20] and the only

pathologic material guiding systemic therapy may be small biopsy and cytology specimens. Until the recent use of TKIs, the standard first-line treatment for most patients with unresectable NSCLC and good performance status has involved the use of a combination of chemotherapy regimens (usually cisplatin-based), which from the 1970s and 1980s were shown to reproducibly achieve objective response in 20–30 % of advanced NSCLC patients. The most common combination regimens in use at present are gemcitabine with either cisplatin or carboplatin, followed by paclitaxel-carboplatin, vinorelbine-platinum and docetaxel-platinum combinations [21, 22]. The addition of the recombinant humanized monoclonal antibody bevacizumab that binds to vascular endothelial growth factor (VEGF) to carboplatin and paclitaxel for the treatment of non-squamous advanced NSCLC has demonstrated to increase RR, PFS and OS when compared to chemotherapy alone [23]. Disease progression affects almost all patients after initial treatment and requires additional therapy. The agents approved for second-line therapy in advanced NSCLC are docetaxel [24], pemetrexed [25] and erlotinib [26]. When tested in randomized trials [24–26], these agents have demonstrated a PFS below 2–3 months with a median overall survival no longer than 9 months in very few unselected patients. Despite recent advances with approval of more active chemotherapeutic and anti-angiogenesis agents for stage IV NSCLC, standard therapy can provide only modest clinical benefits with significant toxicities when used in unselected patients. In 2004, the identification of somatic mutations in the EGFR gene provided the first glimpse of a possible target for a treatment [27, 28] which could maximize clinical outcome in those patients who could benefit from a personalized therapy [29]. This implies the identification of certain characteristic molecular lesions meant to be causally responsible for maintenance of the malignant phenotype and also distinctive of the cancer cells. Therapies targeted to these molecular lesions offer the prospect for tumor control and selectivity with less toxicity than traditional chemotherapy.

Several phase III trials (IPASS; WJTOG 3405; NEJ 002; OPTIMAL; EURTAC) have by now demonstrated dramatic improvement in response rates, quality of life, symptoms, and median progression-free-survival (by 2–5 months) with first-line EGFR-TKI therapy compared with standard platinum-doublet chemotherapy in patients with EGFR mutation-positive NSCLC. Gefitinib and erlotinib were the first two agents to target the tyrosine kinase domain of the EGFR. Both these agents showed encouraging activity in patients with NSCLC who had been previously treated with chemotherapy in the phase I series and then in phase II trials [30, 31]. In order to determine whether an EGFR TKI or chemotherapy is the appropriate first-line therapy, the latest guidelines recommend mutation testing for all patients with advanced NSCLC tumor. All EGFR-mutated patients treated with gefitinib or erlotinib invariably develop acquired resistance to this kind of therapy [32, 33] (Fig. 1). The most common and first identified mutation is the threonine-790 to methionine (T790M) point mutation in exon 20 which represents approximately 50 % of all acquired resistance in NSCLC [34]. The development of such genetic alteration restores the EGFR TK affinity to ATP, rendering first-generation TKIs inactive [35, 36]. Other secondary resistance mutations within the same gene have been reported infrequently (L747S, D761Y,T854A). Other mechanisms of acquired

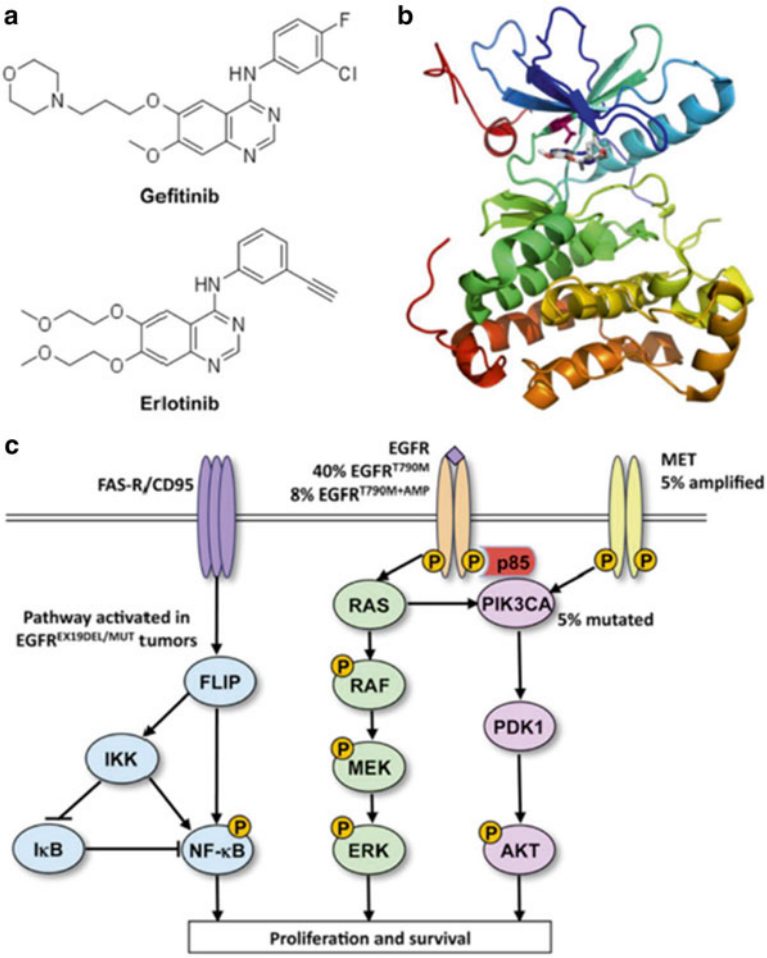

Fig. 1 Ways to leave your EGFR inhibitor: biochemical pathways leading to resistance to small molecule EGFR drugs such as gefitinib and erlotinib. (**a**) Structures of two approved EGFR TKIs, gefitinib and erlotinib, used in the treatment of NSCLC. (**b**) Ribbon diagram of wild-type human EGFR (PDB code 2ITY), illustrating binding of gefitinib to the active site of the kinase. The *magenta ball-stick* (located just above the gefitinib molecule in the active site) indicates the gatekeeper residue (threonine790) that is commonly mutated to methionine (T790M), resulting in reduced inhibitor binding and drug resistance. (**c**) Simplified pathway diagram of EGFR signaling through RAS/MEK/ERK and PI3K/PDK1/AKT indicating the points of mutation/amplification in EGFR TKI resistance as reported by Sequist and colleagues. The resistance mechanisms include the EGFR T790M gatekeeper mutation, amplification of EGFR T790M, MET amplification, and PI3KCA mutation (note that additional epithelial to mesenchymal transition changes and transformation from the NSCLC to the SCLC phenotype also lead to resistance but are not covered by this illustration). The illustration also shows the FAS/NF-kB signaling arm downstream of the FAS death receptor that was shown to be important in TKI resistance by Bivona and colleagues (*Reprinted from Cancer Cell, 19, Paul Workman and Paul A. Clarke, "Resisting Targeted Therapy: Fifty Ways to Leave Your EGFR", 437–440, 2011, with permission from Elsevier*)

resistance include MET gene amplification (also accounting for up to 20 % of pre-treatment tumoral resistances) [37], increased signalling through parallel pathways such as the ones of VEGF [38] and IGF1R [30], mutations and activation of PIK3CA [40] and transformation into a small-cell lung cancer phenotype [41]. Management of EGFR tumor resistance has become the next challenge in order to lengthen these patients' overall survival; identification of the molecular resistance mechanisms will allow for the treatment of TKI-resistant tumors. A new class of drugs, the so-called second-generation TKIs, may be able to overcome the T790M mutation resistant cell. Compared to first-generation TKIs, these molecules show higher affinity for the ATP-binding domain, form an irreversible covalent bond to the ATP-binding site and are able to stimulate other receptors (e.g. HER2). Neratinib (HKI-272), one of the three agents investigated, hasn't shown good RR when tested on patients with known T790M mutation [42], therefore further development of this drug in lung cancer has been halted.

Clinical and Surgical Implications of the EGFR-Mutations Pattern

1. **EGFR mutational profile in the pre-treatment assessment of NSCLC**

As the EGFR mutational profile of NSCLCs is a strong predictor of response to therapy with the highly effective TKIs the most recent algorithms for the management of advanced NSCLCs underline the importance of EGFR molecular testing prior to the initiation of therapy and in particular, EGFR mutations should be sought in those NSCLCs in which they occur most frequently such us in the adenocarcinomas. Due to the recent understanding that histologic typing and *EGFR* mutation status are important for target the therapy in lung adenocarcinoma patients [2] there was a great need for a new classification that addresses diagnostic issues and strategic management to allow for molecular testing in small biopsy and cytology specimens.

All previous WHO classifications have addressed histologic classification primarily based on resection specimens. Since only 30 % of lung cancers are resectable, the vast majority of lung cancer patients present with advanced disease and are diagnosed based on small biopsy and cytology specimens. In 2011, the International Association for the Study of Lung Cancer, American Thoracic Society, and European Respiratory Society proposed a new classification for lung adenocarcinoma that included a number of changes to previous classifications. This classification now considers resection specimens and small biopsies well as cytology specimens. For resection specimens, the new terms of adenocarcinoma in situ and minimally invasive adenocarcinoma are introduced for small adenocarcinomas with pure lepidic and predominantly lepidic growth, with invasion $\leq$5 mm, respectively. Invasive adenocarcinomas are now classified by their predominant pattern as lepidic, acinar, papillary, and solid; a micropapillary pattern is newly added.

This classification also provides guidance for small biopsies due to the recent understanding that histologic typing and EGFR mutation status are important for target the therapy in lung adenocarcinoma patients. Actually, the value of adenocarcinoma in situ or minimally invasive adenocarcinoma for early stage NSCLC and the value of EGFR expressed in patients with advanced NSCLC predicting a benefit in terms of survival.

In the near future more surgical biopsies (in early and advanced disease) may be needed to define the best therapeutic strategy.

2. **EGFR mutational profile in post-treatment assessment of locally-advanced NSCLC**

It seems that chemotherapy is able to modify the EGFR expression in NSCLCs by increasing or decreasing it [44].

This chemotherapy-related change may partially explain why chemotherapy resistant tumors are less sensitive to EGFR-TKI treatment than chemotherapy-naive tumors [45].

Moreover, the modification of the EGFR mutational pattern during chemotherapy may also explain why almost all clinical trials involving second-line TKI therapy have failed to show a positive correlation between EGFR mutation and progression-free or overall survival [26, 46].

Moreover, the shift in tumors from EGFR mutation status to wild-type status observed after first-line chemotherapy suggests that both mutant and nonmutant cancer cells coexist in the same tumor.

To identify intratumor heterogeneity, Bai and co-workers [47] microdissected and analyzed EGFR mutation status in more than 2506 tumor foci of 79 tumors from patients with NSCLC who underwent palliative surgery. Approximately 38 % of tumors contained both EGFR-mutant and wild-type foci. It is interesting to note that a majority of EGFRmutation changes after chemotherapy were from mutant state to wild type, suggesting that cancer cells harboring EGFR mutations might be more sensitive to chemotherapy than those without mutation. Furtherly, Bai and coll. analyzed the relationships between chemotherapy responses and the shift of EGFR mutation status and found patients who achieved PR were more likely to have had EGFR mutation shift than those achieving SD (Stable Disease) or PD (Progression Disease) after chemotherapy.

Therefore, it may be reasonable assumed that EGFR mutation shift could be related to the heterogeneity of intratumoral EGFR mutation and to different chemo-sensitivity levels of mutant and wild-type cells. These findings should be considered in future studies designed to elucidate the predictive role of EGFR mutation in second-line TKI therapy for patients with NSCLC.

Finally, no studies have been reported till now to investigate EGFR mutational pattern before and after chemotherapy administered with induction intent in locally-advanced potentially resectable (Stage IIIa) NSCLC. As the matter of fact, it may be interesting to investigate the effect of induction chemotherapy – usually based on cisplatinum derivates among with gemcitabine- on the EGFR

mutational pattern, this representing an extreme simplified model first of all to evaluate the clonal resistance of neoplastic cells to drugs and also to investigate the biological response of the disease and the theoretical response to TKIs agents in alternative or in combination with surgical resection of the tumor following the induction protocol.

3. **EGFR mutational profile in the evaluation of suspicious second primary NSCLC**

A better acknowledgement of the correlation between EGFR-mutations pattern and clonality in NSCLC may be extremely useful for other clinical scenarios.

As well, the assessment of multifocal lung tumours and the distinction of synchronous primary tumours from intrapulmonary metastases represents an important problem as this decision significantly influences tumour staging and subsequent treatment strategies.

In order to provide a basis for evidence-based treatment decisions in those patients, some Authors [48–50] have analysed the clonal relationship of multifocal NSCLC with indistinguishable histomorphology in a series of NSCLC patients.

In detail, Warth and co-workers have tested KRAS and EGFR mutations using polymorphic short tandem repeat markers in 78 suspicious multifocal NSCLC patients. Despite the limitation of the small sample, these preliminary data suggested a common clonal origin indicative of intrapulmonary metastases in almost two thirds (∼62 %) of the cases, while ∼36 % of multifocal NSCLC displayed unique molecular profiles suggesting separate primary tumours.

Therefore, as already suggested in 2 [51] although the IASLC/ATS/ERS classification recommended testing only patients with advanced adenocarcinomas for epidermal growth factor receptor (EGFR) mutations, we strongly advocate the assessment of EGFR mutations also in patients with synchronous/metachronous primary adenocarcinomas, because the eventual differences in clonality may indeed be a helpful tool for the differential diagnosis of pulmonary metastases vs secondary lung neoplasms.

Conclusion

It is reasonable to suggest that personalized therapy for NSCLC patients should include a genetic assessment of the EGFR mutational status for individual patients.

The appropriate role of an EGFR mutation routine analysis in the treatment of patients with NSCLC continues to evolve. In this context, preliminary evidences have emerged in the last decade, supporting the concept that EGFR mutation assessment may be a useful tool with clinically relevant implications in almost all settings of NSCLC treatment.

Further clinical trials should evaluate the ability of preoperative TKIs to achieve better results than can be obtained with platinum-based chemotherapy in locally advanced EGFRmut(+) NSCLC patients.

Finally, a close cooperation between clinicians, surgeons, molecular biologists and pathologists is crucial for a continuous improvement in the field of NSCLC target therapy.

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Mechanisms of Resistance to EGFR Tyrosine Kinase Inhibitors and Therapeutic Approaches: An Update

Aarif Ahsan

Abstract Resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) in non small cell lung cancer (NSCLC) is mediated by two major mechanisms namely secondary mutation T790M in EGFR and cMET amplification. Other molecular mediators which contribute towards TKI resistance include the activation of compensatory growth signaling, epithelial mesenchymal transition and microRNAs regulating EGFR and cMET levels. In this chapter, we have included the major mechanisms which contribute towards EGFR TKI resistance in NSCLC. Several therapeutic approaches to overcome TKI resistance are also presented which include second and third generation EGFR TKI inhibitors and cMET inhibitors. Further, the rationale to utilize the combination therapies to simultaneously target EGFR and other major oncogene addictive pathway such as ERBB2 and AXL kinase is outlined. Another promising approach to overcome TKI resistance is to potentiate EGFR protein for degradation. These studies will best be utilized when we can identify the oncogene addictions in an individual patient and tailor the therapy/therapies accordingly for the maximum benefits.

Keywords EGFR • Non small cell lung cancer • TKI resistance • cMET • Erlotinib

EGFR Activating (Drug Sensitive) Mutations

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase which belongs to the EGFR family, consisting of four members: EGFR, ERBB2, ERBB3, and ERBB4. Under physiological conditions, binding of ligands (e.g., epidermal growth factor, transforming growth factor-alpha, amphiregulin) activate the tyrosine

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kinase activity of EGFR via homo- or heterodimerization with EGFR family members [1]. In non small cell lung cancer (NSCLC), mutations in EGFR occur in exons encoding the ATP-binding pocket of the kinase domain (exons 18–21). In a cohort of nearly 1200 patients harboring EGFR mutations which are linked to clinical outcomes, more than 145 different types of nucleotide changes have been reported within the EGFR kinase domain [2]. However, the most clinically relevant and extensively studied drug-sensitive mutations are deletions in exon 19 that eliminate a common amino acid motif (LREA) and point mutations in exon 21 that lead to a substitution of arginine for leucine at position 858 (L858R). Together, these two classes of mutations account for approximately 85 % of *EGFR* mutations in NSCLC. They are constitutively active and oncogenic [3, 4] due to the disruption of autoinhibitory interactions in EGFR [5]. Biochemical studies indicate that these mutants preferentially bind to first generation tyrosine kinase inhibitors (TKIs) like gefitinib and erlotinib over ATP, which account for the dramatic response of patients harboring these mutations to TKIs [5, 6]. Other potential drug-sensitive mutations occur at much lower frequency: G719×(3 %), L861×(2 %), [2] and exon 19 insertions (1 %) [7]. The former two were associated with drug sensitivity in the original reports on EGFR mutations [8, 9], whereas the exon 19 insertions were recently reported as drug sensitive [7]. The rarity of clinical data associated with these less frequent mutants has made it more difficult to determine how drug sensitive they are in patients, however new data are emerging.

Mechanisms of Resistance to TKIs

T790M Mutation in EGFR

Despite initial response to EGFR first generation TKIs, patients with mutant *EGFR* NSCLC experience disease progression within 12 months of treatment [10]. The most common mechanism of acquired resistance is the emergence of a secondary mutation in exon 20, T790M, within the catalytic cleft of EGFR. T790M mutations are detected in approximately 50 % of NSCLCs that become resistant to first-generation EGFR TKIs [11]. The T790M mutation was identified in the germline of a family predisposed to NSCLC, indicating an important role in NSCLC genetic susceptibility [12]. An analysis of pretreatment biopsies from NSCLC patients with *EGFR* mutations who subsequently received erlotinib reported that the incidence of double *EGFR* mutations (L858R or exon 19 deletion as well as T790M) was 35 % (45 of 129) with no difference in the initial response to erlotinib (63.6 % versus 72.3 %) in patients with or without T790M mutations. However, those patients showed lower progression free survival where T790M mutation was present [13]. These findings suggest that the T790M mutation may be present in some patients prior to TKI therapy and may be selected during therapy because of the treatment resistance associated with the mutation, suggesting the possibility of intrinsic resistance in these patients. Initially, steric hindrance of TKIs by the

"gatekeeper" T790M mutation has been hypothesized as the basis for T790M induced TKI resistance. Furthermore, the presence of T790M mutation increases the ATP affinity of the oncogenic L858R mutant by approximately fivefold. Therefore, enhanced ATP affinity reduces the ability of reversible TKIs such as gefitinib and erlotinib to effectively compete with ATP binding. These factors lead to a dramatically reduced potency of TKIs in the setting of the L858R and T790M double mutation [6].

cMET Amplification

Amplification of Hepatocyte growth factor receptor (HGFR/cMET), a receptor tyrosine kinase, was detected in up to 20 % of NSCLC patients that developed acquired resistance to gefitinib or erlotinib. Although, cMET amplification can coexist with the EGFR T790M mutation, approximately 60 % of MET amplification is independent of T790M mutation [14, 15]. cMET amplification was originally identified in a laboratory model of gefitinib resistance using HCC827 human EGFR mutant NSCLC cells. In this gefitinib resistant model, cells with EGFR TKI resistance developed dependency on cMET signaling to activate phospho AKT through ERBB3-mediated activation of PI3K signaling in the presence of EGFR TKIs [15]. Additionally, the cMET ligand hepatocyte growth factor (HGF) is also shown to induce gefitinib resistance through activation of cMET-PI3K signaling [16]. As seen in case of T790M mutations, cMET amplification was also observed at a low frequency in NSCLC patients prior to treatment and was associated with the development of acquired resistance to EGFR TKIs [17]. Together these findings suggest that EGFR TKI treatment may select for preexisting cells with cMET amplification to develop EGFR TKI resistance.

Epithelial Mesenchymal Transition (EMT)

Another mechanism which is shown to confer the resistance to TKI is an increase in EMT in NSCLC [10, 18]. In one study, the erlotinib resistant HCC4006 cells were shown to acquire mesenchymal phenotype and exhibited significant down regulation of E-cadherin [19]. EMT in response to TKI conferring resistance was mediated by TGF Beta and IL-6 axis. Other studies suggest the involvement of ERK2 signaling in TGF Beta mediated EMT in non-transformed cells [20–24]. In these tumors, ERK2 amplification was speculated to be responsible for EMT and TKI resistance [25]. In a recent study, authors utilized tumor xenografts with acquired resistance to erlotinib and found alterations in the expression of several genes that are established biomarkers of erlotinib resistance. For example in resistant tumors, elevated levels of COL6A1 (encoding a type IV collagen), HMGA1 and HMGA2 and reduced levels of keratin genes were found. Importantly, a critical mediator of EMT, AXL kinase was found to be dramatically induced by erlotinib resistance [26]. AXL kinase is a tyrosine protein kinase receptor UFO, which is involved in

stimulation of cell proliferation [27]. In the TKI resistant tumors, up regulation of AXL kinase was found to be the second most prevalent mechanism by which resistance occurs followed by T790M mutation. Activation of AXL kinase occurred due to its over-expression as well as up regulation of its ligand GAS6 in the setting of resistant tumors. The up regulation of AXL kinase activity was a part of EMT associated transcriptional program and Vimentin was involved in it. In this study, AXL-overexpressing HCC827 erlotinib resistant cells showed increased migration and adhesion, the properties associated with EMT and the metastatic behavior of tumor cells. These findings are consistent with previous studies showing that over-expression of AXL is associated with increased metastasis in several types of cancers [28]. These findings also advocate that activation of multiple pathways involved in EMT may promote resistance to EGFR TKIs downstream of AXL upregulation. It is well studied that AXL can drive the growth of cancer cells through activation of several oncogenic pathways [28–30]. It will be important to determine the degree to which AXL activation may cooperate with other genetic and genomic alterations to induce resistance to EGFR inhibitors and other molecularly targeted therapies in NSCLC. The loss of E-cadherin can also be mediated by upregulation of Cyclooxygenase 2 (COX-2) metabolite, Prostaglandin E2 (PGE2) [31–33]. PGE2 induces rapid ERK phosphorylayion, reduce E-cadherin levels and upregulate the transcriptional repressors zinc-finger E-box binding homeobox 1 (ZEB-1) and zinc-finger factor Snail homologue 1 (Snail) in NSCLC. COX-2 inhibitors were shown to reverse these effects. Therefore, PGE2 or other inflammatory cytokines in the tumor microenvironment may contribute to EGFR TKI resistance in NSCLC by suppressing E-cadherin expression. These findings also provide a strong rationale for simultaneously targeting EGFR and COX-2 for lung cancer treatment.

Epigenetic Mechanisms

The involvement of specific micro RNAs (miRNAs) in regulating the expression of EGFR and cMET receptor tyrosine kinases and consequently, the metastatic behavior and gefitinib resistance in NSCLC is also reported [34]. miR-221 and miR-222 is shown to be regulated by c-MET and miR-30b, miR-30c, miR-221 and miR222 are regulated by both EGFR and cMET. In response to gefitinib treatment, miR-30b, miR-30c, miR-221 and miR222 get down regulated and consequently, Apoptotic protease activating factor 1 (APAF-1) and B cell lymphoma 2 interacting mediator of cell death (BIM) get up regulated in TKI sensitive cells. In gefitinib resistant cells, the levels of these four miRNAs did not decrease suggesting for their involvement in TKI resistance. Further, cMET inhibitors down regulate miR-30b, miR-30c, miR-221 and miR222 in TKI resistant cells. Taken together, these findings indicate that modulation of miR-30b, miR-30c, miR-221 and miR222 could have therapeutic implications to sensitize TKI resistant tumors. miR-221 and miR-222 has also been shown to regulated Phosphatase and Tensin homolog (PTEN) expression which might contribute towards TKI resistance [35–37]. Loss of PTEN is shown to aberrantly activate EGFR and c-MET signaling.

Targeting the Resistance Mechanisms

Tyrosine Kinase Inhibitors

To overcome the TKI resistance mediated by first generation TKIs, the second and third generation EGFR inhibitors are being developed which are outlined in Table 1.

Second Generations Inhibitors

The development of drugs that bind irreversibly to ERBB family members and/or inhibit multiple targets simultaneously, are being investigated to treat NSCLCs that are resistant to first-generation EGFR TKIs [11]. Unlike reversible TKIs, irreversible TKIs contain a reactive Michael-acceptor group that binds covalently with Cys797 present at the ATP-binding cleft of mutant EGFR. This approach provides greater presence at the ATP site and overcoming the competition with ATP [6, 38]. The ability of an irreversible TKI to overcome resistance was demonstrated in vitro in mutant EGFR cell lines [39]. Several investigational irreversible multitargeted HER family TKIs (Table 1) are being evaluated in patients with NSCLC. These include neratinib or HKI-272 (Wyeth, which was acquired by Pfizer in 2009, New London, CT), PF00299804 (Pfizer), and afatinib or BIBW 2992 (Boehringer Ingelheim, Ingelheim, Germany).

Table 1 Details and status of EGFR TKIs

Drug name	Generic name	Target	Status
Reversible			
ZD1839	Gefitinib	EGFR	Approved
OSI776	Erlotinib	EGFR	Approved
BPI-2009H	Icotinib	EGFR	Approved
TAK-165	Mubritinib	EGFR/ERBB2	Phase I
XL647	NA	EGFR/ERBB2/FLT-4	Phase II
ZD6474	Vandetanib	EGFR/ERBB2/RET	Phase III
GW572016	Lapatinib	EGFR/ERBB2	Preclinical
Irreversible			
EKB-569	Pelitinib	EGFR	Phase I
CI-1033	Canertinib	EGFR/ERBB2/ERBB4	Phase II
HKI-272	Neratinib	EGFR/ERBB2	Phase II
BIBW2992	Afatinib	EGFR/ERBB2/ERBB4	Phase III
PF-00299804	Daconitinib	EGFR/ERBB2/ERBB4	Phase III
Third generation			
CO-1686	NA	EGFR T790M	Phase I/II
WZ4002	NA	EGFR T790M	Preclinical

Neratinib (HKI-272)

Neratinib, an irreversible ERBB family inhibitor that targets EGFR/ERBB1, ERBB2, and ERBB4 [40, 41] (Table 1), was evaluated in a phase I trial of patients with advanced solid tumors [42]. Of 14 evaluable patients with NSCLC, stable disease (SD) for 24 weeks was observed in six (43 %) patients. Despite preclinical data suggesting a role for neratinib in overcoming resistance mediated by T790M [39], no patients with a known T790M mutation responded in another study. Based on overall results, neratinib is no longer in development for NSCLC (http://www.clinicaltrials.gov), although it is being investigated in ERBB-2 positive breast cancer [43].

PF00299804

PF00299804, an irreversible ERBB family inhibitor that targets EGFR/ERBB1, ERBB3, and ERBB4 [44] (Table 1), has demonstrated preclinical activity in gefitinib-resistant NSCLC models both in vitro and in vivo [45]. In a phase I/II trial of PF00299804 in patients with NSCLC who progressed following one or two prior chemotherapy regimens and erlotinib [46], 36 patients with adenocarcinoma and five patients with non-adenocarcinoma histology were evaluated for efficacy. Among patients with adenocarcinoma, 67 % had a clinical benefit (response), and among those with non-adenocarcinoma histology, the clinical benefit rate was 40 %.

Afatinib (BIBW 2992)

Afatinib is an oral irreversible ERBB family inhibitor that targets EGFR/ ERBB1, ERBB2 [47], and ERBB4 with preclinical data supporting a role in overcoming resistance to reversible EGFR TKIs [47]. Afatinib has been studied in multiple phase I clinical trials [47–52]. Three patients with NSCLC experienced PRs lasting 24, 18, and 34 months; their tumors were found to have mutations in EGFR, although none had received prior EGFR TKI treatment. Two additional patients (one with NSCLC and one with esophageal cancer) had unconfirmed partial responses (PRs). One of the NSCLC patients with an activating exon 19 mutation who had a PR was initially treated with afatinib (10 mg/day) but subsequently progressed and developed brain metastases. By investigator assessment, the objective relative risk (RR), disease control rate (DCR), median progression free survival (PFS) interval, and median OS time were 60 %, 86 %, 14 months, and 24 months, respectively, for all patients [49]. The objective RR, DCR, and median PFS were 59 %, 83 %, and 16.1 months, respectively, for patients with L858R mutations and 69 %, 93 %, and 13.7 months, respectively, for patients with exon 19 deletions.

Third Generation (T790M EGFR Specific) Tyrosine Kinase Inhibitors

Despite the initial promise, the 4-anilinoquinazoline core structure that is common to the clinically available irreversible inhibitors, the second generation TKIs, do not show specificity towards T790M EGFR compared with wild-type EGFR. New structurally distinct irreversible ERBB family inhibitors, such as the pyrimidine-based inhibitors described were recently introduced by Zhou et al. [53]. This study screened a library of compounds to identify agents that inhibited growth of gefitinib-resistant and gefitinib-sensitive cell lines without producing toxicity in mutant KRAS cells at high concentrations. One such compound, WZ4002, is an irreversible inhibitor with chemical properties that favor 100-fold greater binding to the T790M mutant. WZ4002 also demonstrated a 100-fold weaker binding to wild-type EGFR than with neratinib and other quinazoline-based second generation EGFR inhibitors. Additionally, WZ4002 inhibited L858R/T790M EGFR kinase activity more potently than wild-type EGFR protein activity, whereas the opposite was true for neratinib and gefitinib. Such findings indicate that the concept of irreversible ERBB family inhibition is a very promising and may yet provide a solution to the problem of acquired resistance.

cMET Inhibitors

Multiple agents that inhibit the cMET signaling at various points have been studied. HGF-competitive analogs, such as NK4, have shown inhibitory activity in various cancer cell lines, [54–59]. Other compounds such as decoy cMET and the isolated Sema domain of cMET have the ability to simultaneously bind to both the ligand HGF and the receptor cMET [60, 61]. These agents have shown inhibition of cMET signaling in preclinical studies. Studies with specific antibodies against HGF/cMET have also shown encouraging results. Monoclonal antibodies against cMET, such as OA-5D5 and DN30, have been shown to cause tumor-cell growth inhibition [62–64]. In addition, monoclonal antibodies against HGF have also been developed (L2G7 and AMG102) and validated in several preclinical studies [65, 66]. Another way to inhibit the cMET pathway is through competitors for the ATP binding site in the TK domain of cMET. This type of inhibition is carried by the small molecule inhibitors such as PHA665752, ARQ 197, SGX523, JNJ-38877605, EXEL-2880, XL-184, MGCD265, MK2461, crizotinib (PF-02341066), K252a and MP470 (Table 2). Engelman et al. exposed the gefitinib resistant cells to the cMET inhibitor PHA665752, and restored tumor sensitivity to gefitinib by reducing EGFR phosphorylation and inducing apoptosis [15, 67]. In the model of immnodeficient mice with enhanced tumor growth due to increased production of HGF, the MET-specific small-molecule kinase inhibitor SGX523 partially inhibits the HGF-dependent growth of lung, breast and pancreatic tumors [68]. So far, the most relevant compound developed in this arena is crizotinib (PF02341066), which has been recently

Table 2 cMET inhibitors under development

Agent	Mechanism of action
PHA665752	Specific cMET inhibitor, ATP competitor
ARQ197	Selective cMET inhibitor, non-ATP competitor
SGX523	Selective cMET inhibitor, ATP competitor
JNJ-38877605	Selective cMET inhibitor, ATP competitor
EXEL-2880	TKI, targets HGF and VEGFR family members
XL-184	Pan TKI
MGLD-265	cMET, VEGFR 1/2/3, Tie 2, Ron
MK-2461	Multi TKIs
K252a	Multi TKIs
MP470	TKI targets cMET, PDGFR, c-Kit

approved by the US FDA for the treatment of EML4/ALK mutant NSCLC tumors, targets cMET and ALK receptor TK. In a panel of different tumor cell lines, crizotinib inhibited phosphorylation of wild-type cMET. In lung carcinoma cells, crizotinib inhibited HGF-stimulated cell migration and invasion [69].

Strategic Combination Therapies

In selected cases combination therapies have been utilized, specifically to inhibit the compensatory pathway which is activated and mediates TKI resistance.

Combining EGFR and c-MET Inhibitors

For tumors harboring cMET amplification as a determinant to cause TKI resistance, targeting cMET receptor in combination to EGFR inhibitors are likely to predict a better response compared with individual targeting of EGFR. Antibodies targeting the cMET ligand, antibodies targeting MET itself and small molecules inhibitors against cMET are the therapeutic options for these combination therapies. The simultaneous inhibition of EGFR and cMET was shown to suppress the proliferation of cells and anti-tumor efficacy in mice in HCC827 cells which develop gefitinib resistance [15, 17]. Another study was carried out in NCI-H820 cells which naturally harbor EGFR T790M mutation as well as cMET amplification. In this study, small molecule cMET inhibition or knockdown of cMET along with EGFR inhibition suppressed the compensatory ERBB3 signaling and compromised cell viability [14]. From these studies, it was not clear whether T790M EGFR and cMET amplification co-occur in the same cell and whether the cell type is dependent on both of these factors. To address these issues, Xu et al. carried out a study, where they

developed mouse model of adenocarcinoma harboring both T790M EGFR and cMET. In this study treatment with individual inhibitors of EGFR and cMET was un-affective and the combinatorial targeting of both these receptors caused significant tumor regression. Importantly, this study strengthened the notion that in tumors harboring both T790M and cMET amplifications, both these lesions are drivers for growth [70].

EGFR and ERBB2 Inhibitors

The rationale of combining EGFR and ERBB2 inhibitors is via various molecular interactions across their downstream signaling pathways. It is known that the ligand independent activation of EGFR can be mediated by ERBB2 amplification. Overexpression and amplification of ERBB2 decreased the degradation of EGFR and increases its recycling to the cell membrane [71]. For simultaneous targeting of EGFR and ERBB2, two classes of inhibitors have been developed: agents which bind reversibly and those that bind irreversibly (covalently) to the ATP binding site in the tyrosine kinase domain in EGFR and ERBB2. Due to the mechanisms governing the resistance to reversible TKI, irreversible inhibitors targeting both EGFR and ERBB2 are likely to be better therapeutic choices. Irreversible TKI such as BIBW 2992 is shown to inhibit the autophosphorylation of EGFR and ERBB2. This agent was more than 100-fold potent that gefitinib against cells harboring the T790M+L858R mutation [47]. Another irreversible inhibitor HKI-272 caused dramatic tumor regression in mice model of TKI resistance [72]. In a Phase I clinical trial of BIBW2992, out of 26 patients with adenocarcinoma, 2 showed partial response. Another Phase II single arm clinical trial using BIBW2992, recently reported partial response in 43 patients out of 67 in mutation positive patients. The disease control rate was 96 % and a median progression free survival was 10.2 months. A clinical trial utilizing HKI-272 showed stable disease in 42 % of 16 NSCLC patients previously treated with gefitinib.

EGFR and EMT Inhibitors

To overcome the EMT associated with TKI resistance several mediators have been targeted [73]. Due to the role of ERK1/2 in EMT, ERK1/2 blockade by U0126 led to a suppression of TGF-Beta mediated EMT in NSCLC, restored epithelial phenotype of these cells and sensitized the TKI resistant cells in combination with gefitinib [74]. E-cadherin re-expression is also utilized to target EMT in overcoming TKI resistance. ZEB1 is known to down regulate E-cadherin and promote EMT. ZEB1 was shown to be inhibited by utilizing Histone deacetylase inhibitors. In this study, the combination of HDAC inhibitors with erlotinib led to the reversal of TKI resistance in HCC4006ER cells [19]. Since the up regulation of AXL kinase was shown to be a critical determinant of TKI resistance in NSCLC, targeting of AXL kinase sensitized HCC827ER cells. In this study, knockdown of AXL kinase

in HCC827 parental cells did not affect survival, however, the knockdown decreased the survival of HCC827ER cells, suggesting the specific role of AXL kinase in mediating erlotinib resistance in these cells. Small molecule inhibitors of AXL kinase, MP-470 and XL-880 [28] in combination with erlotinib also decreased the viability of HCC827ER cells. The process of EMT is also governed by inflammatory signals [75]. The inflammatory enzyme COX-2 is frequently over-expressed in a variety of malignancies [76]. COX2 plays an important role in conferring malignant and metastatic phenotypes and its over-expression is involved in therapy resistance [77]. For instance, COX-2 over-expression in NSCLC is associated with apoptosis resistance, [78] angiogenesis, [79, 80] and metastasis [81, 82]. Most of these effects are mediated by prostaglandin E2 (PGE2). PGE2 and other inflammatory cytokines in the tumor microenvironment may contribute to TKI resistance by downregulating the levels of E-cadherin. These findings suggest a strong rationale of combining COX inhibitors with TKI to overcome TKI resistance [83]. However the clinical trials combining COX2 inhibitors with EGFR inhibitors did not show additional benefits compared with the individual treatment of EGFR inhibitor. It seems possible that in these trials sufficient dose of COX2 inhibitor was not used to inhibit maximum COX2 activity [84, 85].

EGFR and mTOR Pathway Inhibitors

The rationale for combining EGFR inhibitors with mTOR inhibitors was based on studies suggesting an important survival function of mTOR-AKT axis as downstream effector of EGFR signaling [86]. The ability of the irreversible EGFR inhibitor HKI-272 and rapamycin combination to promote more effective suppression of EGFR signaling to S6 and AKT kinases was demonstrated both in cultured NSCLC cell lines harboring double mutant EGFR alleles as well as in lung tumors in TL mice. The investigators suggest that HKI-272 may not sufficiently overcome the biochemical drug resistance conferred by T790M and that further suppression of an essential AKT-mTOR signal downstream of EGFR is required to achieve a therapeutic response [87].

Promoting Oncogene Degradation

An interesting aspect by which TKI resistance has been targeted is via EGFR degradation. It is now well accepted that EGFR degradation is superior in causing cancer cell cyto-toxicity compared to simply inhibiting its tyrosine kinase activity. Weihua et al. reported that knockdown of EGFR caused autophagy and inhibition of EGFR only caused a transient cell cycle arrest [88]. Other findings in head and neck tumor models suggest that promoting EGFR degradation may lead to cell death [89, 90]. Chemotherapeutic agents such as gemcitabine and cisplatin caused EGFR degradation which correlated with cell death. The importance of targeting EGFR for degradation to overcome TKI resistance was shown by knocking down EGFR in

several EGFR mutant cell lines. In this study, knockdown of EGFR caused a decrease in survival of EGFR dependent cells including NCI-H1975 cells harboring EGFR-T790M mutation [91]. This approach was utilized recently to develop a peptide based therapy which caused EGFR degradation in TKI resistant cells harboring T790M mutant EGFR. The peptide, named as Disruptin was shown to decrease survival of TKI resistant NCI-H1975 cells by disrupting EGFR-Heat shock protein 90 interaction, inhibiting EGFR homodimerization and promoting EGFR degradation [92].

Conclusions and Future Perspectives

The main signaling pathways contributing towards TKI resistance and the therapeutic approaches to rationally target them are summarized in Fig. 1. In order to better target the EGFR TKI resistance, it will be critical to understand the driver oncogene mediating resistance in a specific patient. The prescreening of patients for the driver mutations and amplifications need to be carried out and the personalized therapies need to be tailored depending upon the driver oncogene. For example, in patients with AXL kinase amplification or COX2 over-expression, AXL or COX2 inhibitors

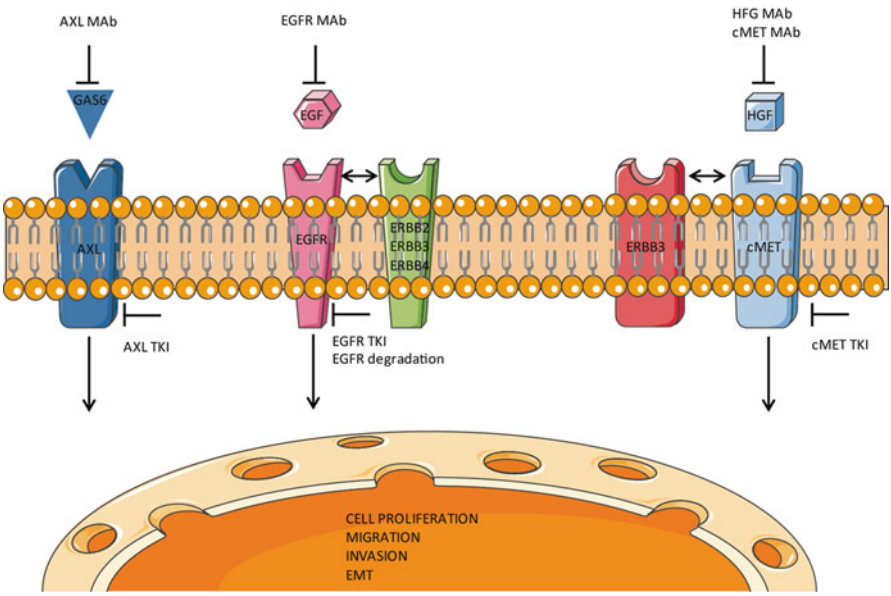

Fig. 1 Major signaling pathways which contribute towards the resistance to EGFR TKIs. In addition to EGFR family members, ERBB2, ERBB3 and ERBB4, AXL and cMET kinases have been reported to mediate EGFR TKI resistance in NSCLC patients. These compensatory pathways can be co-targeted by small molecule inhibitors and antibodies in combination with EGFR TKIs to overcome resistance as shown in the figure

need to be combined with EGFR TKI. Several preclinical studies suggest the re-emission of resistance due to another compensatory mechanism in response to individual therapies. As for instance, in case of irreversible EGFR inhibitors, resistance develops due to downstream signaling mediators. A more effective approach would be to combine the first generation EGFR inhibitor with the second or preferably third generation TKIs. Another promising approach is to target EGFR for degradation, which accounts for inhibition of other functions of EGFR apart from its tyrosine kinase activity which are important for cancer cell survival. These elusive functions of EGFR might be governing the activation of compensatory pathway or downstream signaling mediators imparting TKI resistance. Since the majority of NSCLC patients which harbor TKI sensitive mutations also contain T790M mutation, combining the T790M specific third generation inhibitors in combination with first generation inhibitors as a combined therapy could be a preferred therapeutic choice for these tumors. The Holy Grail to maximize the benefit of each NSCLC patient with EGFR activating mutation is to identify the oncogene addiction/s along with EGFR and provide a tailored combination therapy at the beginning of treatment before the resistance develops.

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KRAS-Mutant Lung Cancers in the Era of Targeted Therapy

Jarushka Naidoo and Alexander Drilon

Abstract *KRAS*-mutant lung cancers account for approximately 25 % of non-small cell lung carcinomas, thus representing an enormous burden of cancer worldwide. *KRAS* mutations are clear drivers of tumor growth and are characterized by a complex biology involving the interaction between mutant KRAS, various growth factor pathways, and tumor suppressor genes. While *KRAS* mutations are classically associated with a significant smoking history, they are also identified in a substantial proportion of never-smokers. These mutations are found largely in lung adenocarcinomas with solid growth patterns and tumor-infiltrating lymphocytes. A variety of tools are available for diagnosis including Sanger sequencing, multiplex mutational hotspot profiling, and next-generation sequencing. The prognostic and predictive roles of *KRAS* status remain controversial. It has become increasingly clear, however, that *KRAS* mutations drive primary resistance to EGFR tyrosine kinase inhibition. Until recently, mutant *KRAS* was not thought of as a clinically-targetable driver in lung cancers. With the expansion of our knowledge regarding the biology of *KRAS*-mutant lung cancers and the role of MEK and PI3K/mTOR inhibition, the face of targeted therapeutics for this genomic subset of patients is slowly beginning to change.

Keywords KRAS mutation • Lung cancer • Lung adenocarcinoma • Erlotinib resistance • Targeted therapy • MEK inhibition • Selumetinib • PI3K inhibition • mTOR inhibition • Hsp90 inhibition

Introduction

It is without question that the discovery of driver oncogenes has revolutionized the field of lung cancer therapeutics. Since the description of activating mutations in the epidermal growth factor receptor (*EGFR*) in 2004 and the demonstration of dramatic efficacy of EGFR tyrosine kinase inhibition in large randomized trials,

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treatment paradigms have quickly shifted towards an emphasis on molecularly-targeted therapy in genomic subsets of patients. With the increase in scope and sensitivity of molecular diagnostic testing, a putative driver is now identified in the majority of lung cancer patients.

KRAS mutations are perhaps the most infamous of these genomic aberrations. While these drivers were first reported in lung cancers in the mid-80s and remain the most commonly mutated oncogenes in unselected patients, they have risen to fame as elusive targets in a rapidly-evolving field of molecular therapeutics [1]. As our knowledge regarding the biology of *KRAS*-mutant lung cancers grows, the latter is slowly beginning to change. Recent data on the efficacy of combinatorial therapy in *KRAS*-mutant lung cancers has sparked renewed interest in developing therapies for patients with these tumors.

In this chapter, we discuss the biology of *KRAS* mutations and tackle their predictive and prognostic roles in lung cancers. We then go on to detail the various molecularly-targeted strategies that have been employed to treat patients with these cancers.

The Biology of RAS

Activated RAS (short for 'rat sarcoma') proteins are key regulators of cell growth across a variety of malignancies. They act to integrate signals from external growth factors with a variety of downstream effectors such as members of the RAF-MEK-ERK and PI3K-AKT-mTOR pathways [2].

RAS proteins are farnesylated via post-translational modifications. This results in localization to the cell membrane that is thought to be essential for activity. RAS proteins shuttle between inactive and active states, during which they are bound to GDP and GTP, respectively. These states are depicted in Fig. 1. GDP bound to inactive RAS is exchanged for GTP by GEFs or guanine nucleotide exchange factors, thus resulting in RAS activation [3]. Conversely, in activated RAS, the hydrolysis of GTP to GDP results in signal termination. This step is catalyzed by GAPs or GTPase-activating proteins [4]. In addition, RAS proteins have intrinsic GTPase activity. GTP-bound activated RAS engages effector molecules belonging to multiple signal transduction cascades that, in addition to cell proliferation and survival, control other processes such as apoptosis, cell cycling, motility, and endocytosis.

Three different RAS genes have been described in humans: *KRAS*, *HRAS*, and *NRAS*. The former two were first identified in the 1960s in studies of cancer-causing sarcoma viruses, namely the Kristen sarcoma virus and the Harvey sarcoma virus. *NRAS* was subsequently identified in human neuroblastoma cells. Activating point mutations in *RAS* lead to mutant RAS proteins that acquire transforming potential. This occurs secondary to impaired GTPase activity and constitutive activation of signaling.

Structurally, these mutations result in replacement of an amino acid at position 12, 13, or 61 [5]. The standard nomenclature used to describe these mutations denotes an amino acid change that occurs at one of these positions. For example,

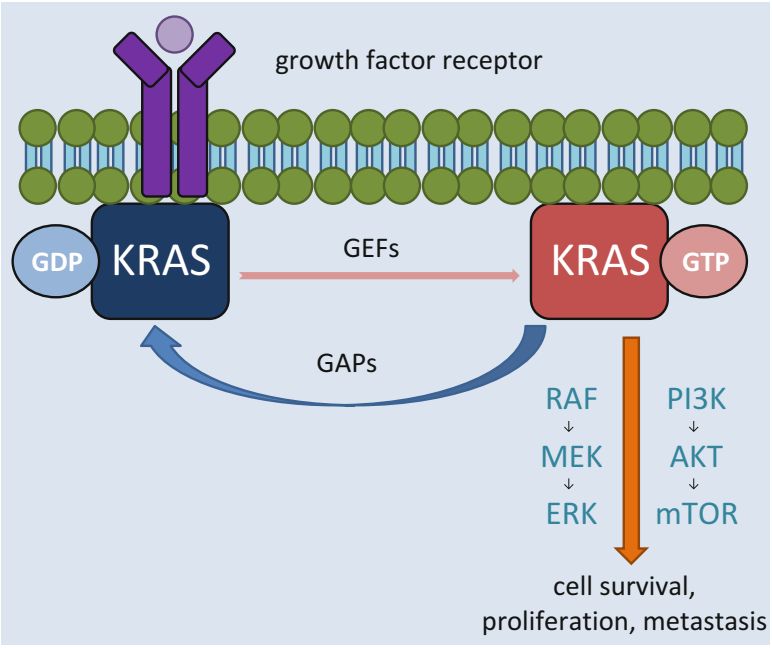

Fig. 1 KRAS signaling. KRAS acts to integrate external signals from extracellular ligands that bind transmembrane growth factor receptors. The KRAS protein is localized to the cell membrane via farnesylation and shuttles between inactive and active states in response to these upstream signals. In its inactive state, KRAS is bound to GDP. Guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP, thereby activating KRAS. Activated KRAS results in increased downstream signaling of growth factor pathways involved in cellular proliferation, survival, and metastasis (e.g. RAF-MEK-ERK and PI3K-AKT-mTOR). GTPase-activating proteins (GAPs) hydrolyze GTP to GDP and result in signal termination

KRAS G12D refers to a point mutation resulting in the substitution of the amino acid glycine (G) that is found in the wild-type state at position 12 of the KRAS protein, with the amino acid asparate (D). Preclinical work in *KRAS*-mutant lung cancer cell lines has suggested that the type of amino acid substitution (e.g. G12C vs G12D) may affect downstream signaling differently, leading to a differential response to cytotoxic therapy [6]. Further studies are required to confirm this hypothesis.

KRAS-Mutant Lung Cancers: Clinicopathologic and Molecular Features

KRAS mutations account for the majority of RAS mutations in human malignancies. They are implicated in the pathogenesis of a variety of solid tumors. *KRAS* mutations have been identified in 60–90 % of pancreatic cancers, 35 % of colorectal cancers, 20 % of serous ovarian cancers, and 15 % of thyroid cancers [7].

KRAS mutations were first described in lung cancers in 1984 [8]. Santos et al. reported that an activating *KRAS* mutation was found in a human lung cancer specimen and not in normal tissue from the same patient, demonstrating that the mutation was somatically acquired. We have since come to learn that *KRAS* is the most commonly mutated oncogene in non-small cell lung carcinomas (NSCLCs), occurring with a frequency of approximately 25 % of unselected cases [9].

A variety of molecular diagnostics are used to identify mutations in *KRAS*. Testing can involve standard Sanger sequencing of the *KRAS* gene, or multiplex testing for specific mutational hotspots (e.g. Sequenom or SnapShot platforms). While these diagnostics have been extremely valuable over the last few decades, we are quickly moving into an era of massively-parallel high throughput or next-generation sequencing. In contrast to mutational hotspot testing that interrogates only specific mutations in *KRAS*, next generation sequencing affords the advantage of both the identification of mutations along the length of the gene and elucidation of aberrations in other tumor suppressor genes or oncogenes (Table 1).

KRAS mutations are largely found in lung adenocarcinomas although they have been infrequently described in squamous cell lung carcinomas where rigorous pathologic review was conducted [10, 11]. Rekhtman and colleagues tested 180 lung adenocarcinomas for mutations in *KRAS* and *EGFR* and assessed these

Table 1 ***KRAS*-mutant lung cancers**. Methods used to diagnose *KRAS* mutations in non-small cell lung cancer specimens are listed here. In addition, the various clinicopathologic and molecular features associated with *KRAS*-mutant lung cancers are described

KRAS mutations in non-small cell lung cancers	
Diagnosis	Traditional Sanger sequencing Multiplex mutational hotspot profiling Sequenom SnapShot Next-generation sequencing
Clinical features	Classically associated with a significant history of current or former smoking While less common, can be found in a substantial proportion of never or former light smokers Reported to be more common in Caucasians compared to Asians
Pathologic features	Found largely in lung adenocarcinomas Pathologic associations Solid growth pattern Mucinous tumors Tumor-infiltrating leukocytes
Molecular features	Tend to be mutually exclusive with other lung cancer drivers (e.g. *EGFR* mutations, *ALK* fusions) Can coexist with various tumor suppressor gene aberrations Point mutation profile varies **Transitions** (G12D, G13D, G12S): more common in never or former light smokers **Transversions** (G12C, G12A, G12V, G13C): more common in patients with a significant smoking history

specimens for the proportion of standard histologic patterns (acinar, lepidic, solid, mucinous, papillary, and micropapillary). In comparison to *EGFR*-mutant and *KRAS/EGFR*-wild-type tumors, the solid growth pattern was significantly over-represented in *KRAS*-mutant tumors. *KRAS*-mutations were also more commonly seen in mucinous adenocarcinomas that were significantly associated with the presence of tumor-infiltrating leukocytes [12].

Classically, *KRAS* mutations are thought to be found more commonly in patients with a significant history of smoking [13, 14]. In 2008, however, work by Riely et al. established that *KRAS* mutations are found in up to 15 % of never-smokers with lung cancers. We are now aware that this phenomenon extends to other drivers in lung cancers. While driver oncogenes can be found more commonly in patients with specific smoking histories (e.g. *ALK* fusions in never-smokers, *BRAF* mutations in current or former smokers), molecular testing should not be withheld from patients regardless of their pack-year history as these aberrations are identified in patients with varying degrees of exposure to tobacco. There is some evidence to support ethnic differences in the frequency of KRAS mutations, with a higher frequency noted in Caucasians compared to Asians and potentially African Americans [15, 16].

While exceptions to the rule have been reported, *KRAS* mutations tend to be mutually exclusive with other known lung cancer drivers such as *EGFR* mutations and *ALK* fusions. Substantial heterogeneity exists in the specific type of point mutation found in *KRAS*. In an analysis of data from the Catalogue of Somatic Mutations in Cancer (COSMIC), G12C was the most common mutation (42 %), followed by G12V (21 %), and G12D (17 %) [5]. A number of other mutations in codons 12, 13, and 61 have been described at lower frequencies.

Similar to what has been described with *TP53*, the type of point mutation found in *KRAS*-mutant lung cancers varies by smoking history. Transition point mutations (where a purine is exchanged for a purine or a pyrimidine for a pyrimidine, e.g. A→G or T→C, respectively), are more commonly found in never-smokers. The transition *KRAS* G12D is the most common point mutation in never-smokers. Other examples of transitions include G13D and G12S. Transversion point mutations (where a purine is exchanged for a pyrimidine or vice-versa, e.g. G →C or A→T), on the other hand, are more common in former/current smokers. *KRAS* G12C is the most common transversion in this population [17, 18]. Other examples of transversions include G12A, G12V, and G13C.

The Prognostic Nature of *KRAS* Mutations

Soon after the description of *KRAS* mutations in lung cancer in 1984, a number of studies began to emerge addressing the potential prognostic nature of these aberrations. In the 1990s, the prevailing sentiment was that *KRAS* mutations represented a negative prognostic marker for survival in patients with lung adenocarcinomas [19]. This viewpoint has since come into question as data from both individual and pooled studies have generated conflicting results.

Table 2 ***KRAS* status as a prognostic marker in lung cancer**. Selected individual and pooled studies investigating the role of *KRAS* mutations as potential prognostic markers in non-small cell lung cancers are summarized here. While *KRAS* mutations were thought to be negative prognostic factors for survival, data remains conflicting

Reference	Patients tested for *KRAS*	Patients by *KRAS* status		Results *KRAS*-Mt vs *KRAS* WT
		Mt	WT	
Selected individual studies				
Kern et al. 1994 [20]	n = 44 Stage I–IV	16 (36 %)	28 (64 %)	HR for OS 1.7 (0.8–3.5), p = 0.16
Keohavong et al. 1996 [21]	n = 173 Stage I–IV	43 (25 %)	140 (75 %)	No difference in OS, p = 0.96
Graziano et al. 1999 [22]	n = 213 Stage I–II	35 (16 %)	178 (84 %)	Median OS 39 mo vs 53 mo, p = 0.33
Schiller et al. 2001 [9]	n = 184 Stage II–IIIA	44 (24 %)	140 (76 %)	Median OS 30 mo vs 42 mo, p = 0.38
Lu et al. 2004 [23]	n = 94 Stage I	32 (34 %)	62 (66 %)	HR for OS 1.18 (0.71–1.95), p = 0.52
Grossi et al. 2003 [24]	n = 249 Stage I–IIIA	47 (19 %)	202 (81 %)	HR for OS 1.46 (0.96–2.22), p = 0.08
Tsao et al. 2007 [25]	n = 450 Stage IB–II	117 (26 %)	333 (74 %)	HR for OS 1.23 (0.76–1.97), p = 0.40
Pooled analyses				
Mascaux et al. meta-analysis, 2005 [26]	n = 3620 Stage I–IV	18 % by PCR	82 % by PCR	HR for OS 1.35 (1.16–1.56), p = 0.01 (*KRAS* mutation or p21 expression)
Shepherd et al. LACE-Bio, 2013 [27]	n = 1,532 Stage I–III	300 (19 %)	1,232 (80 %)	HR for OS 1.17 (0.96–1.42), p = 0.12

Mt mutant, *WT* wild-type, *HR* hazard ratio, *PFS* progression-free survival, *OS* overall survival, *mo* months

Individual Studies The role of *KRAS* mutations as a prognostic factor in lung cancer remains a controversial issue. While the prognostic nature of *KRAS* status has been studied widely in non-small cell lung carcinomas of all stages, patients with early-stage lung cancers represent a significant proportion of subjects. In comparison to studies in advanced-stage lung cancers, surgical specimens in early-stage studies afford the advantage of larger tumor samples on which molecular diagnostic testing can be more easily performed. These studies are summarized in Table 2.

The Eastern Cooperative Oncology Group (ECOG) E4592 study was a randomized control trial investigating the potential benefit of adjuvant thoracic radiation with or without four cycles of cisplatin/etoposide in patients with resected stage II-IIIA non-small cell lung cancers. This was the first large prospective study that assessed the role of *KRAS* mutations as determinants of prognosis. 184 tumors in this study were evaluated for *KRAS* status. Of the 44 *KRAS*-mutant tumors, 33 % were of non-squamous histology and 4.8 % were squamous cell carcinomas. While the overall survival of patients with *KRAS*-mutant lung cancers was numerically

inferior to patients with *KRAS* wild-type lung cancers, this finding was not statistically significant. The median overall survival of patients with *KRAS*-mutant tumors was 30 months, compared to 42 months for patients with KRAS wild-type tumors (p = 0.38). On multivariate analysis, only age and tumor stage were found to be significant prognostic factors, although a trend was observed bordering on statistical significance for KRAS status (p = 0.07) [9].

In the JBR.10 study of patients with resected stage IB-II NSCLC, patients were randomized to receive either four cycles of adjuvant cisplatin/vinorelbine or observation. This was a positive trial that demonstrated a 15 % absolute improvement in 5-year survival in patients who received chemotherapy versus observation (HR, 0.78; 95 % CI, 0.61–0.99; p = 0.04). Overall, *RAS* mutations were detected in 24 % of patients, and this finding was not prognostic for overall survival (p = 0.40) [28].

The International Agency for Research on Cancer (IACR) examined the effect of *KRAS* status in early stage resected NSCLCs in the European Early Lung Cancer trial. *KRAS* mutations were identified in 18.5 % of tumors, 30.6 % of which were lung adenocarcinomas (n = 41/134) and 4.3 % squamous cell carcinomas (n = 5/115). *KRAS* status was not shown to be prognostic for progression-free survival (p = 0.26) [29].

The Cancer and Leukemia Group B-9633 (CALGB-9633) trial was a phase III study that randomized patients with stage IB NSCLC to either four cycles of adjuvant carboplatin/paclitaxel or observation. The study was a negative trial that failed to meet its primary endpoint. *KRAS* mutations were detected in 27 % of tumors (n = 71/258), and no prognostic effect of *KRAS* status was demonstrated (HR for OS 1.1, p = 0.747) [30].

Pooled Analyses The individual studies discussed in the previous section have largely been limited by small numbers and insufficient power to draw conclusions on the prognostic nature of *KRAS* status in non-small cell lung cancers. Thus, a number of meta-analyses have been performed in attempt to answer this question.

Mascaux and colleagues published a meta-analysis of 28 studies and 3620 patients aimed at assessing the prognostic significance of *KRAS* status on both disease-free survival and overall survival in the adjuvant setting. The study demonstrated that the presence of a *KRAS* mutation was a negative prognostic factor for overall survival (HR for OS 1.35, CI 1.16–1.56, p = 0.01, random effect model), although this took into account both patients with a *KRAS* mutation and p21 expression. Similarly, *KRAS* mutations portended poorer overall survival in patients with lung adenocarcinomas (HR 1.52, CI 1.30–1.78, p = 0.02). These findings were not seen in the subset of patients with squamous cell lung carcinomas (HR 1.49, CI 0.88–2.52, p = 0.48). *KRAS* status was a significant prognostic marker when polymerase chain reaction sequencing was employed as the method of assessment (HR 1.39, CI 1.22–1.58, p = 0.03). In contrast, immunohistochemistry (IHC) to detect p21 status was not found to be significantly prognostic (1.08, CI 0.86–1.34, p = 0.21) [26].

Pooled data from 1721 patients from four randomized control trials comparing adjuvant chemotherapy versus observation (ANITA, IALT, JBR.10, and

CALGB-9633) were analyzed as part of the LACE-Bio (Lung Adjuvant Cisplatin Evaluation-Biomarker) study. *KRAS* status was tested by restriction fragment length polymorphism, allelic specific oligonucleotide hybridization, or allelic refractory mutation system analysis and mass spectrometry. These detection methods were employed as they were deemed to demonstrate greater sensitivity than direct sequencing techniques [27].

KRAS status was successfully determined in 1532 patients. 20 % of these samples were found to be *KRAS*-mutant, of which 34 % were adenocarcinomas (n = 206/602), 6 % were squamous cell carcinomas (n = 44/705.) and 23 % were from non-squamous, non-adenocarcinoma histologies (n = 53/229.) *KRAS* mutations were found to be more frequent in women, younger patients, and early stage disease. In a multivariate analysis, only age ($p = 0.04$) and histology ($p < 0.0001$) were significant prognostic indicators.

There was no significant difference in overall survival based on *KRAS* status (HR for OS 1.17, CI 0.96–1.42, $p = 0.12$) with no heterogeneity among trials ($p = 0.47$). Similarly, there was no significant difference in disease-free survival based on *KRAS* status (HR for DFS 1.15, CI 0.96 = 1.39, $p = 0.14$). No prognostic difference for overall survival was demonstrated between different types of *KRAS* mutations, such as those involving codon 12 (HR: 1.04, CI 0.77–1.40) or codon 13 (HR 1.01, CI, 0.47–2.17, $p = 0.96$). There was no significant difference in prognosis for codon 12 subgroups for both disease-free ($p = 0.98$) and overall survival ($p = 0.99$).

The Predictive Nature of *KRAS* Mutations

Benefit of Adjuvant Chemotherapy in Early-Stage Lung Cancers A number of randomized studies have investigated whether *KRAS* status might predict for response to adjuvant chemotherapy in non-small cell lung cancers. Similar to what we have seen in the previous section on the prognostic value of *KRAS* status, these trials have demonstrated discordant results. To date, *KRAS* status has not served as a valuable criterion to determine if patients with resected non-small cell lung cancers should receive adjuvant platinum-based chemotherapy.

In the JBR.10 study of adjuvant cisplatin and vinorelbine in resected stage IB-II non-small cell lung cancers, no statistically significant benefit of chemotherapy over observation was observed in patients with *RAS* wild-type tumors (median survival 7.8 vs 6.6 years, HR, 0.84, CI 0.63–1.12; $p = 0.24$). Similar results were obtained for patients with *RAS*-mutant lung cancers (median survival 9.7 vs 7.8 years, HR 0.82, CI 0.50–1.35, $p = 0.44$). Although the interaction term was non-significant for disease-specific survival, *RAS* wild-type patients appeared to derive more benefit from chemotherapy (HR 0.72, CI 0.51–1.02, $p = 0.06$) compared with *RAS*-mutant patients (HR, 1.07, CI 0.61–1.88, $p = 0.82$) [28].

In the CALGB 9633 study of adjuvant carboplatin and paclitaxel for resected stage IB non-small cell lung cancers, 5-year overall survival was not significantly

different between *KRAS*-mutant and *KRAS* wild-type patients that received chemotherapy (55 % vs 62 %, HR 1.2, p = 0.58). Five-year overall survival was likewise not different between *KRAS*-mutant and *KRAS* wild-type patients who were randomized to observation (67 % vs 59 %, HR 1.1, p = 0.75). Adjuvant chemotherapy in patients with large tumors (>4 cm) was not significantly associated with benefit in patients with *KRAS* wild-type (HR 0.69, p = 0.18) or *KRAS*-mutant patients (HR 1.2, p = 0.55) [30].

Given the limitations of previous studies, investigators pooled data from three Lung Adjuvant Cisplatin Evaluation (LACE) platinum-based adjuvant chemotherapy trials to examine the role of *KRAS* status. The analysis revealed no significant effect of *KRAS* status on overall survival benefit from adjuvant chemotherapy over observation (*KRAS* wild-type tumors HR 0.89, CI 0.76–1.05, p = 0.15; *KRAS*-mutant tumors HR 1.05, CI 0.76–1.46, p = 0.77). Results were not different among trials (p = 0.52). Results were similar for disease-free survival (*KRAS* wild-type tumors HR 0.86, CI 0.74–1.00, p = 0.04; *KRAS*-mutant tumors HR 0.93, CI 0.68–1.27, p = 0.65) [27].

In terms of the different types of *KRAS* mutations, no benefit in overall survival was seen in patients with codon 12 mutations (HR 0.95, CI 0.67–1.35, p = 0.77). However, patients with codon 13 mutations had worse outcomes with adjuvant chemotherapy compared to patients who did not receive chemotherapy (HR for OS 5.78, CI 2.06–16.2, p < 0.01). A variable effect on overall survival was seen with codon 12 mutations: G12A or G12R (HR 0.66, p = 0.48), G12C or G12V (HR 0.94, p = 0.77) and G12D or G12S (HR 1.39, p = 0.48) but these differences were not significant (comparison of four HRs, including wild-type, p = 0.76). The authors concluded that *KRAS* status cannot be used to select patients with non-small cell lung cancer for adjuvant chemotherapy.

Benefit of Chemotherapy in Advanced Lung Cancers Little is known regarding the role of *KRAS* mutations as predictors of response or resistance to cytotoxic chemotherapy. As with adjuvant chemotherapy, currently available data do not support the use of *KRAS* status as a means of selecting patients for systemic chemotherapy.

In the phase III TRIBUTE (Tarceva Responses in Conjunction with Paclitaxel and Carboplatin trial in advanced NSCLC) trial, first-line chemotherapy with carboplatin, paclitaxel, and erlotinib was compared to carboplatin, paclitaxel, and placebo. *KRAS* mutations were present in 21 % of tumor samples tested. In patients that received carboplatin and paclitaxel alone, response rate was not different between patients with *KRAS*-mutant and *KRAS* wild-type tumors (23 % vs 26 %). Time to progression and overall survival in the *KRAS*-mutant and *KRAS* wild-type cohorts that received chemotherapy alone were as follows: TTP 6 mo vs 5.4 mo, OS 13.5 mo vs 11.3 mo [31].

Small retrospective studies have been reported that did not reveal statistically significant benefits for palliative chemotherapy based on *KRAS* status in patients with advanced non-small cell lung cancers [32, 33]. In a retrospective study by Levy and colleagues of 16 patients with *KRAS*-mutant/*EGFR*-wild-type and 19 patients with *KRAS*/*EGFR*-wild-type non-small cell lung cancers who received first-line

platinum-based pemetrexed-containing chemotherapy, overall response rate was not significantly different (56 % vs 36 %, p=0.30). Within the limits of a small retrospective series and variable follow-up, median progression-free survival was improved in *KRAS*-mutant patients vs *KRAS* wild-type patients (10.3 vs 5.7 mo, p=0.03). Overall survival was not reported [34]. These results remain hypothesis-generating.

Benefit of EGFR-Directed Targeted Therapy The KRAS protein lies directly downstream of the epidermal growth factor receptor (EGFR). Due to its position in the signaling cascade, mutant KRAS is hypothesized to cause persistent pathway activation independent of EGFR signaling, thus conferring resistance to EGFR tyrosine kinase inhibition (TKI). While activating *EGFR* mutations are recognized as the strongest predictors of benefit from EGFR TKI use, a number of trials in advanced non-small cell lung cancers have asked the question of whether or not *KRAS* mutations are negative predictors of response to EGFR TKI therapy (Table 3).

The potential role for *KRAS* status as a predictive marker, was investigated in two phase III clinical trials examining single agent EGFR TKI versus best supportive care. The NCIC BR.21 trial compared erlotinib with placebo in patients with stage IIIB/IV non-small cell lung cancers who received one or two prior chemotherapy regimens. Overall survival was improved from 4.7 to 6.7 months in patients who were randomized to placebo versus erlotinib (p<0.01). No significant difference in response rate was noted in patients who received erlotinib with *KRAS*-mutant vs

Table 3 ***KRAS* status as a predictive marker of EGFR TKI benefit**. Selected individual and pooled studies investigating the role of *KRAS* mutations as potential predictive markers of EGFR tyrosine kinase inhibition (TKI) with gefitinib or erlotinib in non-small cell lung cancers are summarized here. In general, *KRAS* mutations are thought to confer resistance to therapy

Study	Arm	Endpoint	*KRAS*-Mt	*KRAS* WT
TRIBUTE Eberhard et al. [31]	Carboplatin, paclitaxel, and erlotinib	ORR	8 %	26 %
		TTP	3.4 mo	5.3 mo
		OS	4.4 mo	12.1 mo
Hirsch et al. [35]	Erlotinib	ORR	7 %	19 %
		PFS	3 mo	3 mo
		OS	12 mo	11 mo
Massarelli et al. [36]	Erlotinib or gefitinib	ORR	0 %	13 %
		TTP	1.7 mo	2.4 mo
		OS	5.0 mo	9.4 mo
TRUST Schneider et al. [37]	Erlotinib	ORR	0 %	9 %
BR.21 Zhu et al. [38]	Erlotinib	ORR	5 %	10 %
INTEREST Doulliard et al. [39]	Gefitinib	ORR	0 %	10 %
		PFS	1.4 mo	2.6 mo
		OS	7.8 mo	7.5 mo
Mao et al. pooled [14]	Erlotinib or gefitinib	RR	3 %	26 %

Mt mutant, *WT* wild-type, *HR* hazard ratio, *PFS* progression-free survival, *OS* overall survival, *TTP* time to progression, *mo* months

KRAS wild-type tumors (5 % vs 10 %, p = 0.69). An interaction test did not demonstrate a significant difference in survival based on *KRAS* status (interaction $p = 0.09$) [38]. A similar analysis was undertaken for the ISEL study (Iressa Survival Evaluation in Lung Cancer) of gefinitinib vs placebo in second- and third-line patients with advanced non-small cell lung cancers. A *KRAS* mutation was detected in 7.9 % ($n = 12$) of 152 tumor samples. Due to the limited number of cases detected, no reliable conclusion could be drawn from the impact of *KRAS* status on the benefit of gefitinib versus best-supportive care [35]. A number of other studies examining the role of *KRAS* status as a predictive marker of benefit from EGFR TKI inhibition are summarized in Table 3.

The TRIBUTE trial supports the potential role for *KRAS* as a negative predictor of response to erlotinib plus chemotherapy versus chemotherapy alone. In patients with *KRAS*-mutant tumors, overall response rate was lower in patients who received erlotinib and chemotherapy versus those that received erlotinib alone (ORR 8 % vs 23 %). Patients with *KRAS*-mutant tumors who were treated with erlotinib and chemotherapy had a shorter median time to progression (TTP 3.4 mo, CI 1.5–6.3) and overall survival (OS 4.4 mo, CI 3.4–12.9) compared to patients who received chemotherapy alone (TTP 6 mo, CI 4.9–7.1; OS 13.5 mo, CI 11.1–15.9). Among patients with *KRAS*-mutant tumors, the hazard ratio of erlotinib plus chemotherapy versus chemotherapy alone was 2.1 (CI 1.1–3.8) for OS and 1.9 (CI 1.1–3.6) for TTP [31].

Two meta-analyses have assessed the association between *KRAS* status and response to EGFR TKI in non-small cell lung cancers. Linardou and colleagues pooled data from 17 non-small cell lung cancer trials, representing a total of 165 patients with *KRAS* mutations. In this analysis, *KRAS* mutations were significantly associated with an absence of response to EGFR tyrosine kinase inhibition (sensitivity 0.21, specificity 0.94, positive likelihood ratio 3.52, negative likelihood ratio 0.84). A pooled sensitivity analysis demonstrated that no response was seen in some *KRAS* wild-type tumors, leading the authors to believe that resistance to EGFR TKIs is unlikely to be solely mediated by *KRAS* mutation status (0.21; 95 % CI: 0.16–0.28) [40].

Data from 22 trials in non-small cell lung cancer was pooled for a meta-analysis by Mao and colleagues. 16 % of these patients (n = 231/1470) harbored tumors with mutant *KRAS*. The response rate to EGFR tyrosine kinase inhibition was 26 % in patients with *KRAS* wild-type tumors compared to 3 % for *KRAS*-mutant tumors. The pooled relative risk for response was 0.29 (95 % CI: 0.18–0.47; $p < 0.01$). This analysis was mirrored in both Asian and Caucasian patients, with a relative risk of 0.22 in Asians (95 % CI: 0.07–0.63; p = 0.01) and 0.31 in Caucasians (95 % CI: 0.17–0.54; $p < 0.01$) [14].

No validated data exists regarding *KRAS* mutation type and differential response to EGFR tyrosine kinase inhibition. A small study (n = 14) by Metro and colleagues demonstrated that patients with codon 13 mutations had worse progression-free (p = 0.04) and overall survival (p < 0.01) compared to patients with codon 12 mutations [41].

Patients with advanced non-small cell lung cancers treated with the EGFR monoclonal antibody cetuximab were studied in the BMS099 and FLEX (First-Line Erbitux) trials [42, 43]. In BMS099, patients were randomized to either carboplatin

and taxane chemotherapy alone or with cetuximab. *KRAS* mutations were found in 17 % of tumors in this study, and no statistically significant association between *KRAS* status and response rate, progression-free survival, or overall survival was demonstrated [42]. The FLEX trial compared palliative cisplatin and vinorelbine with or without cetuximab in EGFR-expressing non-small cell lung cancers. *KRAS* mutations were detected in 19 % of tumors and were not significantly associated with response outcomes [43]. These findings are contrasted to data generated in colorectal cancers where *KRAS* mutations are predictive of poor response to cetuximab and panitumumab.

Therapeutic Targeting of *KRAS*-Mutant Lung Cancers

Cytotoxic chemotherapy remains the current standard of care for patients with advanced *KRAS*-mutant non-small cell lung cancers. While *KRAS* mutations in lung cancer have been known to us over the last three decades, many efforts at targeting these drivers have not yielded palpable results. Mutant KRAS plays a crucial role in the integration of extracellular growth signals with downstream signaling cascades, and approaches to targeted therapy have focused on inhibiting several steps along these pathways.

The landscape of potential targeted therapeutics for mutant KRAS, however, is slowly beginning to change as evidenced by reports of the efficacy of MEK inhibition in combination with chemotherapy. As our knowledge of the biology of *KRAS-mutant* lung cancers continues to expand, this space is likely to undergo significant evolution over time.

This evolution will involve a more granular understanding of synthetic lethal reactions that lead to cell death in *KRAS*-mutant lung cancers. While the more common KRAS-targeting strategies are discussed in this section (Figs. 2 and 3), other potential avenues of research include studies of the NF1, WT1, EZH2, Siah 2, GATA2, RBM5, IL-8, TWIST1, cyclin-dependent kinase, and propapoptotic pathways [5]. It is worth emphasizing that the biology of *KRAS*-mutant lung cancers is highly complex and involves a delicate interplay between various tumor suppressors and oncogenes.

Farnesyl Transferase Inhibition Earlier efforts at targeting KRAS in lung cancers focused on inhibiting mutant RAS with antisense oligonucleotides against RAS itself. Problems with administration and drug delivery have however, halted further studies in this area. Investigators then began to focus on inhibiting the association of mutant KRAS with the plasma membrane as this event was thought to be crucial for activity. The KRAS protein associates with the plasma membrane via a process called farnesylation. This step is catalyzed by the enzyme farnesyl transferase, resulting in the post translational modification of the C-terminal CAAX motif of KRAS by the addition of a farnesyl isoprenoid lipid [44].

Farnesyl transferase inhibitors (FTIs) were thus developed with the intent of abolishing signal transduction and cell growth via prevention of localization mutant

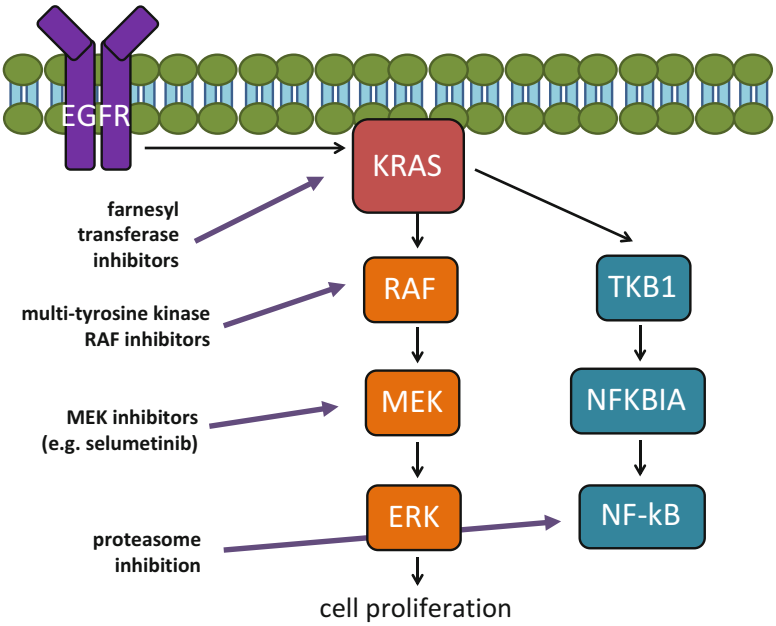

Fig. 2 Therapeutic targeting strategies in *KRAS*-mutant lung cancers. Mutant KRAS lies downstream of growth factor receptors such as EGFR. Constitutive activation of mutant KRAS results in subsequent activation of downstream signaling of downstream pathways (RAF-MEK-ERK, and NF-kB). Targeted therapies directed against various members of these pathways are depicted

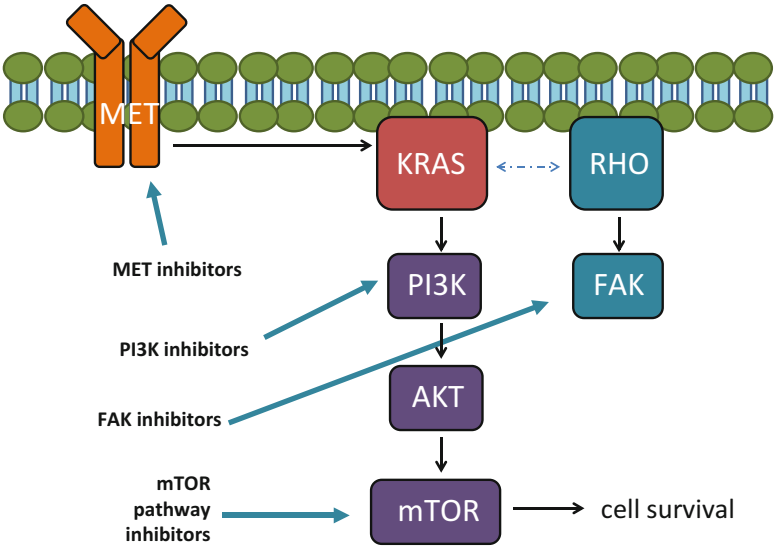

Fig. 3 Other strategies to target *KRAS*-mutant lung cancers. In addition to activation of the RAF-MEK-ERK and NF-kB pathways, *KRAS*-mutant lung cancers can be reliant on activation of MET or the PI3K-AKT-mTOR and RHO-FAK pathways. The various inhibitors used to potentially target these pathways are depicted

KRAS to the cell membrane. These drugs were developed based on rational design strategies and screening of combinatorial libraries. A number of FTIs have been tested including tiparfinib (R115777), lonafarnib, and FTI-277. In vitro experiments revealed activity against KRAS substrates in the low nanomolar range, and in vivo activity was demonstrated in chemically-induced *KRAS*-mutant lung tumors in mice [45, 46]. Unfortunately, the results of clinical testing of farnesyl transferase inhibitors in lung cancer have not yielded significant results.

Despite a large number of clinical trials investigating the use of these drugs as single agents, FTIs have not demonstrated significant activity against *KRAS*-mutant tumors. R115777 was studied by Adeji and colleagues in the first-line setting in a phase II study of an unselected population of patients with advanced NSCLC. The compound demonstrated minimal clinical activity and no objective responses were observed. Seven patients (16 %, CI 8–31 %) achieved stable disease for a period longer than 6 months [47].

Riely and colleagues investigated the FTI salirasib in a phase II trial of 30 patients with *KRAS*-mutant advanced non-small cell lung cancers. Again, no radiographic responses were observed. 30 % (n = 7/20) of patients achieved stable disease at 10 weeks. It is unclear whether the addition of standard chemotherapy to an FTI would result in greater activity, however, results in single-agent studies have been discouraging [48].

RAF Inhibition As RAF proteins lie immediately downstream of mutant KRAS, RAF inhibition was thought to represent a reasonable method of targeting *KRAS-mutant* lung cancers. Many RAF inhibitors that are available in the clinic are multi-tyrosine kinase inhibitors that lack specificity for RAF alone. Initial studies of these 'dirty' tyrosine kinase inhibitors included drugs such as sorafenib. In vitro work in non-small cell lung cancer cell lines with *KRAS* mutations revealed that sorafenib inhibited cell growth and induced G1 arrest secondary to C-RAF depletion [49].

Clinically, the use of sorafenib in patients with *KRAS*-mutant lung cancers was investigated in a single-arm phase II study. Patients with stage IIIB/IV non-small cell lung carcinomas with *KRAS*-mutations who had previously progressed on at least one platinum-containing regimen were treated with 400 mg of sorafenib twice daily until progression or unacceptable toxicity. The primary endpoint of the study was disease control rate at 6 weeks. A total of 57 patients with *KRAS*-mutant lung cancers were treated on this study. A disease-control rate at 6 weeks of 53 % was achieved, however only 5 of 57 patients (9 %) had a partial response to therapy [50].

While this study demonstrated clinical activity of the drug in this genotypic subset of lung cancers, the observed response rate was poor in comparison to the efficacy seen with other targeted therapeutics in driver-positive lung cancers where response rates well exceeding 60 % are noted. In addition, it is unclear whether the effects seen in this population were truly secondary to RAF inhibition versus the inhibition of other targets of the drug including VEGFR and PDGFR.

Drugs thought to have better activity against RAF such as vemurafenib and dabrafenib have since been tested widely in a variety of cancers including advanced *BRAF*-mutant melanomas where their use is FDA-approved. The utility of these

drugs in lung cancer has largely been limited to patients whose tumors harbor *BRAF* mutations, drivers that tend to be mutually exclusive with mutations in *KRAS*.

MEK Inhibition Similar to RAF, MEK lies downstream of KRAS in the RAS-RAF-MEK-ERK pathway. While RAF inhibitors are relatively non-specific, MEK inhibitors such as selumetinib were developed as allosteric inhibitors of the MEK protein via non-ATP-competitive binding and are relatively specific for MEK1 and MEK2.

Davies et al. performed in vitro screening for the activity of selumetinib (also known as AZD6244 or ARRY-142886) against a variety of cancer cell lines and found that cell lines harboring RAS or BRAF mutations were most sensitive to the drug [51]. Ji and colleagues studied the MEK inhibitor CI-1040 in a KRAS G12D-mutant murine lung cancer mouse model [52]. Treatment with CI-1040 resulted in a dramatic reduction of tumor burden (53 % ± 5 %) and histologic analysis of four of five mice revealed they were completely free of tumor. Tumor shrinkage was thought to be secondary to growth arrest and induction of apoptosis [52].

In a phase II study by Hainsworth and colleagues (n = 84), an unselected population of patients with advanced non-small cell lung cancers were randomized to receive either selumetinib at 100 mg orally twice daily or pemetrexed 500 mg/m^2 intravenously once every 3 weeks in the second or third-line setting [53]. No benefit in median PFS was seen between the two arms (67 vs 90 days, HR 1.08, CI 0.75–1.54, p = 0.79). Response rates were also similar between the arms (5 % vs 5 %). *KRAS* status was not reported in this series [53].

Preclinical work by Chen et al. established that the efficacy of docetaxel in *KRAS*-mutant lung cancers can be improved by the addition of a MEK inhibitor. The group demonstrated in vivo that loss of the tumor suppressor genes *TP53* and *LKB1* impaired the response of *KRAS*-mutant lung cancer mouse models to docetaxel. Response rates were significantly increased with the addition of selumetinib to docetaxel. In contrast, however, response in *KRAS*-mutant tumors with concomitant mutations in *TP53* and *LKB1* was significantly lower, emphasizing the importance of concomitant tumor suppressor gene alterations as co-determinants of efficacy of targeted therapy [54].

Janne and colleagues thus investigated the combination of selumetinib and docetaxel versus docetaxel alone in a phase II randomized study of *KRAS*-mutant non-small cell lung cancers in the second-line setting [55]. Forty-four patients received selumetinib and docetaxel, and 43 patients received docetaxel alone. A statistically significant improvement in response rate was seen in the selumetinib-containing arm compared to docetaxel monotherapy (32 % vs 0 %, p < 0.01), accompanied by a significant improvement in progression-free survival (HR 0.58, CI 0.42–0.79, p = 0.01). Median overall survival was not statistically different between the two arms (HR 0.80, CI 0.56–1.14, p = 0.21). Selumetinib is currently being investigated in combination with erlotinib in patients with *KRAS*-mutant tumors [56].

Trametinib (also known as GSK1120212) is a novel MEK inhibitor that has demonstrated activity in preclinical models [57]. This compound was investigated in the phase II setting in 30 heavily pre-treated patients with advanced non-small cell lung cancers. 22 patients of these patients had *KRAS*-mutant tumors [58]. Best radiologic

responses consisted of two patients with partial remission and 10 patients with stable disease. The median progression-free survival in the *KRAS*-mutant subgroup was 3.8 months (95 % CI 1.9–5.5) versus 2.1 months (95 % CI 1.8–5.2) in the *KRAS* wild-type group [58]. This compound is under investigation as a single agent compared to docetaxel in *KRAS*-mutant non-small cell lung cancers.

NF-kB Inhibition In an RNA interference screen of human cell lines, Barbie et al. demonstrated that knockdown of TKB1, an IkB kinase (IKK) that enhances NF-kB activity, resulted in the selective death of cells that harbor *KRAS* mutations [59]. In a separate paper, Meylan et al. demonstrated that the introduction of a nonphosphorylatable NF-kB super repressor, IkB, in *KRAS* G12D-mutant murine tumors resulted in loss of cell viability secondary to apoptosis [60]. This data supports the dependence of *KRAS* G12D-mutant tumors on the NF-kB pathway.

Proteasome inhibition by bortezomib leads to decreased NF-kB signaling by preventing degradation of the NF-kB inhibitor IkB [61]. Luo et al. demonstrated that both shRNA targeting of proteasome subunits and proteasome inhibition by bortezomib resulted in synthetic lethality in *KRAS*-mutant cells [62]. In $KRAS^{LSL\text{-}G12D/wt};p53^{flox/flox}$ mice (G12D-mutant), bortezomib induced in-vivo tumor regression of lung adenocarcinoma. After repeated treatment, however, acquired resistance invariably developed. Similar results were noted with Bay-117082, an NF-kB inhibitor [63].

These preclinical observations provide a compelling rationale to test the use of bortezomib in *KRAS*-mutant lung adenocarcinomas. The use of subcutaneous bortezomib is currently being studied in a phase II study of patients with advanced *KRAS*-mutant non-small cell lung cancers.

MET Inhibition MET aberrations are perhaps most well known in lung cancers in the setting of acquired resistance to EGFR tyrosine kinase inhibition where *MET* amplification or overexpression is found in a subset of patients. MET has likewise been implicated in the cellular transformation induced by mutant RAS. Yang and colleagues studied the role of MET inhibition with PHA-665752 in a *KRAS*-mutant lung cancer mouse model. Short-term treatment with the drug induced apoptosis in tumor cells [64].

Sequist et al. investigated the activity of tivantinib (ARQ 197), a small molecule with activity against MET, in combination with erlotinib in a phase II study. Patients were randomized to erlotinib with or without tivantinib. Overall, the addition of tivantinib failed to improve progression-free survival (HR 0.81, CI 0.57–1.16, $p=0.24$), however a significant benefit in progression-free survival was demonstrated in the patients with KRAS-mutant tumors ($n=15$, HR 0.18, CI 0.05–0.70, $p<0.01$, interaction $p<0.01$) [65].

The MARQUEE study, a subsequent randomized phase III trial with a similar design randomizing patients to erlotinib with or without tivantinib, was stratified by *EGFR* and *KRAS* status. While an interim analysis demonstrated a significant progression-free survival benefit in the intention- to-treat population, the study was closed early by the Data Monitoring Committee as achievement of the primary endpoint of overall survival was unlikely to be met [66]. A subgroup analysis of the

patients with *KRAS*-mutant tumors from this study will be of interest and has yet to be reported.

FAK Inhibition *KRAS* mutations can occur in combination with inactivation of *CDKN2A* via mutations or epigenetic modifications. *CDKN2A* encodes the tumor suppressor genes ARF and INK4A. In combination, mutations in *KRAS* and deficiency of *CDKN2A* have been associated with aggressive non-small cell lung cancers. Konstantinidiou and colleagues reported that *CDKN2A* loss in *KRAS* G12D-mutant lung tumors resulted in aberrant activation of the small GTPase RHOA that is involved in the regulation of chemotaxis and cell migration. RHOA activation is driven by MEK1/2 and ERK/12 and is essential for cell survival [67].

While no specific drugs target RHOA, the group showed that knockdown of the downstream target focal adhesion kinase or FAK resulted in loss of cell viability. Similarly, the administration of the ATP-competitive FAK inhibitor PF562271 resulted in significant tumor regressions in vivo in a *KRAS* G12D-mutant and INK4A- and ARF-deficient model. An ongoing phase II study is looking at the use of the FAK inhibitor V2-6063 (defactinib) in patients with *KRAS*-mutant lung cancers.

PI3K Pathway Inhibition The PI3K (phosphatidyl 3-kinase) pathway is an important growth pathway in cancer cells that is responsible for cell survival, differentiation, motility, and proliferation. Preclinical evidence points to the reliance of *KRAS*-mutant lung cancers on PI3K pathway (PI3K-AKT-mTOR) activation in cooperation with activation of the RAS-RAF-MEK-ERK axis. PI3K signaling is thought to be essential for *KRAS*-induced tumorigenesis but not tumor maintenance.

Engleman and colleagues studied the effects of the dual pan-PI3K and mammalian target of rapamycin (mTOR) inhibitor NVP-BEZ235 in genetically-engineered mouse models. No tumor shrinkage was observed when *KRAS* G12D-mutant transgenic mice were treated with NVP-BEZ235 despite a decreased in AKT phosphorylation. Treatment with selumetinib, however, produced only modest tumor regression. In contrast, treatment with both NVP-BEZ235 and selumetinib resulted in marked synergistic tumor regression and pathologic analysis at the end of treatment revealed a substantial pathologic response [68].

A number of phase I trials are now looking at a combination of MEK inhibition with PI3K-directed therapy, and radiographic responses have been reported by independent investigators. These findings will require validation in later-phase studies. In addition, however, the additive toxicity of two targeted therapies remains a significant concern.

mTOR Inhibition The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that acts downstream of the PI3K/AKT pathway. mTOR inhibitors are thought to achieve their anti-tumor effect by arresting the tumor cells in the G1 phase of the cell cycle [69].

The mTOR inhibitor ridaforolimus was investigated in advanced KRAS-mutant non-small cell lung cancers in a phase II randomized discontinuation study in the second and third-line settings. Patients in this study received 8 weeks of ridaforolimus at 40 mg daily on a 5 day per week schedule and were subsequently randomized to

receive either ridaforolimus or placebo if they achieved stable disease. Overall response rate after 8 weeks of ridaforolimus was 1 %. The primary endpoint of progression-free survival after randomization was significantly improved to 2–4 months in patients who received ridaforolimus versus placebo (HR 0.36, p = 0.013). Median overall survival was 18 months in the ridaforolimus group compared to 5 months in the placebo group, although this finding was not statistically significant (HR: 0.46; p = 0.09) [70].

Heat Shock Protein Inhibition Heat shock proteins are chaperones that play a role in the post-translational modification of clients such as proteins encoded by mutated driver oncogenes, thereby promoting maintenance of the oncogenic phenotype [71]. Acquaviva and colleagues examined the activity of the Hsp90 inhibitor against a panel of lung cancer cell lines with a spectrum of *KRAS* mutations (G12, G13, and Q61 variants). Ganetespib potently reduced viability in all lines with IC50 values in the low nanomolar range. The drug resulted in a dose-dependent decrease in known Hsp90 clients such as EGFR and MET, and CRAF. This was accompanied by inactivation of MEK, ERK, and AKT and the induction of apoptosis [72].

Ganetespib has been investigated as a single agent in a phase II study of unselected non-small cell lung cancers. Fourteen patients were found to have tumors that harbored a *KRAS* mutation, of which one patient achieved partial response and seven patients demonstrated stable disease lasting greater than 16 weeks [73]. A phase IIB/III study combining ganetespib with docetaxel is ongoing, and will investigate potential synergy with these agents.

In the same study by Acquaviva, the combination of low doses of ganetespib with either the MEK inhibitor selumetinib or the PI3K/mTOR inhibitor NVP-BEZ235 resulted in a substantial increase in cell death compared to when the latter two targeted agents were used alone [72]. An ongoing phase Ib/II study is currently evaluating the role of the Hsp90 inhibitor retaspimycin (IPI-504) in combination with the mTOR inhibitor everolimus in the treatment of *KRAS*-mutant non-small cell lung cancers.

Conclusions

In the ever-changing world of targeted therapy for molecular drivers of lung cancer growth, *KRAS* mutations continue to represent a significant challenge for drug development. Despite advances in our understanding of the role of MEK and PI3K/mTOR inhibition in this genomic subset of tumors, responses have largely been observed with combination therapy and not with single agents. This is unlike the paradigm established for other drivers such as *EGFR* and *BRAF* mutations, and *ALK* and *ROS1* fusions where dramatic and durable responses occur with a single targeted therapeutic. Furthermore, combination therapy is plagued by an increase in treatment-related toxicities compounded by the length of time that these agents are used. Our challenge moving forward will be to find agents that are either active as

monotherapy, or relatively safe when used in combination with other targeted therapeutics. This can only be achieved by a more nuanced understanding of the intricate biology that drives these tumors.

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Anaplastic Lymphoma Kinase (ALK) Signaling in Lung Cancer

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Abstract Chromosomal rearrangement in the anaplastic lymphoma kinase (*ALK*) gene was identified as an oncogenic driver in non-small cell lung cancer (NSCLC) in 2007. A multi-targeted ALK/ROS1/MET inhibitor, crizotinib, targeting this activated tyrosine kinase has led to significant clinical benefit including tumor shrinkage and prolonged survival without disease progression and has been approved by US FDA since 2011 for the treatment of advanced *ALK*-rearranged NSCLC (Ou et al. Oncologist 17:1351–1375, 2012). Knowledge gained from treating *ALK-rearranged* NSCLC patients including the presenting clinicopathologic characteristics, methods of detecting *ALK*-rearranged NSCLC, pattern of relapse and acquired resistance mechanisms while on crizotinib, and the clinical activities of more potent ALK inhibitors has led us to a detailed and ever expanding knowledge of the ALK signaling pathway in lung cancer but also raising many more questions that remained to be answered in the future. This book chapter will provide a concise summary of the importance of ALK signaling pathway in lung cancer. Understanding the ALK signaling pathway in lung cancer will likely provide the roadmap to the management of major epithelial malignancies driven by receptor tyrosine kinase rearrangement.

Keywords Anaplastic lymphoma kinase rearrangement non-small cell lung cancer • Receptor tyrosine kinase fusion positive tumors • Crizotinib • ALK breakapart FISH • Chromosomal rearrangement

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Molecular Basis of *ALK* Rearrangement in NSCLC

Chromosomal rearrangements have long been recognized as oncogenic drivers in hematological malignancies. Although it has been predicted in early 2000 that chromosomal rearrangements will be found in solid malignancies there was no reports of such rearrangements well into the mid 2000s [1]. This all changed in 2007 when two research groups independently discovered the chromosomal rearrangement in the anaplastic lymphoma kinase gene in non-small cell lung cancer [2, 3]. These two initial reports revealed in NSCLC there was both intrachromosomal deletion and inversion of the echinoderm microtubule associated protein like 4 (EML4) gene to ALK on chromosomal 2 generating an EML4-ALK fusion protein containing the N-terminal portion of EML4 protein that retains the "coil-coiled" dimerization and the C-terminal portion of ALK which contains the kinase domain that is present in the cytoplasm and constitutively active [2]. Further elegant experiments demonstrated that transgenic mice expressing EML4-ALK in the lung results in multiple adenocarcinomas in the lung [4]. ALK is one of 58 human receptor tyrosine kinase receptors (RTKs) [5] and is the namesake rearrangement in anaplastic large cell lymphoma [6]. Although there are multiple pathways could be activated by the constitutively EML4-ALK [7], the common sequala of the ALK activation is the increase in survivin level and a decrease in pro-apoptotic BIM protein allowing lung cancer cells driven by ALK activation to escape apoptosis [8]. Since 2007, several different breakpoints in the *EML4* have been identified that have been fused to the *ALK* generating several EML4-ALK fusion variants [9]. Currently, there are three main EML4-ALK fusion variants. The most common variant among EML4-ALK fusion is variant 1 (54.5 %) followed by variant 3 (34 %) and variant 2 (10 %) [9, 10]. Additionally, other fusion partners to ALK kinase domain have been identified in NSCLC including TFG (Trk-fused gene), KIF5B (kinesin family member 5), and KLC1 (kinesin light chain 1) [9]. As recently as in 2014 new fusion partner, HIP1 (Huntington interacting protein 1), to ALK in NSCLC is continuously being discovered [11, 12]. Indeed any protein that contains a "coil-coiled" domain that can serve as a dimerization domain and can localize to the cytoplasm can potentially act as a fusion partner to ALK. Hence it is likely more fusion partners to ALK in lung cancer will be identified in the future. Hence *ALK*-rearranged NSCLC is a molecularly heterogeneous subset of lung cancer. In vitro experiments have shown that there are potential differential responses among the various EML4-ALK fusion variants to various ALK inhibitors [13]. Therefore is reasonable to hypothesize that these various ALK fusion variants will have subtle but different clinicopathologic characteristics and likely differential response to ALK inhibitors. Additionally activation of epidermal growth factor receptor (EGFR) mutations have been identified in *ALK-rearranged* lung cancer; however, whether activation *EGFR* mutations and/or *ALK* rearrangement is the main oncogenic driver remained to be determined [9].

Clinicopathologic Presentations of *ALK*-Rearranged Lung Cancer

Despite being a molecularly heterogeneous disease, several general patterns can be ascribed to *ALK*-rearranged NSCLC. First, the median age of diagnosis of *ALK-rearranged* NSCLC is in the early to mid 50s which is about 15 years younger than the typical age of diagnosis of lung cancer in the US [14–16]. However the age of diagnosis can range from the early twenties to the eighties subtly underlining this is indeed a molecularly heterogeneous disease [15, 16]. Second, the vast majority (>95 %) of the *ALK*-rearranged lung cancer is adenocarcinoma [15, 16] with a portion of these adenocarcinoma also had signet ring features although signet ring features is not pathognomonic for *ALK* rearrangement [14, 17]. However, case reports have shown that ALK rearrangement has also been found in squamous cell carcinoma [11, 12, 18]. Interestingly, a recent report has shown that *EML4-ALK* fusion transcript is found in small cell lung cancer [19]. Thus as we broaden our screening of *ALK* rearrangement in all histologies of lung cancer, we may gain further knowledge about *ALK* rearrangement in lung cancer. Third, similar to lung cancer driven by activating *EGFR* mutations, between two-thirds to three quarters of *ALK-rearranged* lung cancer patients were never-smokers (defined as <100 cigarettes lifetime) [20]. Therefore, smoking status should not be used as a clinical criterion to determine whether to screen for *ALK* rearrangement in lung cancer. Fourth, unlike *EGFR* mutated lung cancer there does not seem to be a propensity for *ALK-rearranged* lung cancer to be more common in Asian patients although whether a specific variant of EML4-ALK is more prevalent among a certain racial/ethnic group remains to be determined [9]. Fifth, in terms of tumor burden and sites of metastasis at the time of initial presentation, there seems to be some differences between *ALK*-rearranged lung cancer and *EGFR* mutated lung cancer [21]. One particular interests among clinical oncologists treating *ALK*-rearranged lung cancer is whether there is an increase incidence of brain metastasis. This question arises from the observation that close to half of *ALK*-rearranged lung cancer patients who progressed on crizotinib had brain relapses. It does not seem that patients with *ALK-rearranged* lung cancer inherently presents with higher incidence of brain metastasis at the time of diagnosis [21].

Biology of *ALK*-Rearranged Lung Cancer

Our aggregate understanding of the biology of *ALK*-rearranged lung cancer come from differential responses *ALK*-rearranged lung cancers to various chemotherapy agents, the diagnostics methods used to identify *ALK*-rearranged lung cancer patients, patterns of relapse on crizotinib, acquired resistances during crizotinib treatment, and the clinical activity of more potent ALK inhibitors in clinical development.

A randomized trial (PROFILE1007) comparing crizotinib to single chemotherapy agent (docetaxel or pemetrexed) as second line treatment *ALK*-rearranged lung cancer have clearly demonstrated that crizotinib conferred statistically significant progression-free survival (PFS) [22]. However, the response rate of *ALK*-rearranged lung cancer patients treated with pemetrexed was much higher than those treated with docetaxel [22]. This observation is consistent with case series that reported ALK-rearranged lung cancer patients had a higher response rate to pemetrexed [23–26] likely due to a lower level of thymidylate synthase (TS) [26], which is a target of pemetrexed, in the *ALK*-rearranged tumor. It has been postulated that the aberrant activation of ALK lead to repressed transcription of the TS gene.

The current FDA approved companion diagnostic test which was approved simultaneously with the approval of crizotinib in August, 2011 was the Abbott Vysis breakapart fluorescence in situ hybridization (FISH) assay [9, 27]. However FISH is expensive, labor intensive, and requires certain technical competence to interpret the FISH signals [9]. Since 2011, it is becoming clear that immunohistochemistry (IHC) is a equally effective but much cheaper way to detect *ALK* rearrangement in lung cancer [28]. ALK IHC test using a highly sensitive antibody D5F3 can now be automated taking the observer variability out of the testing component [29]. ALK protein is not expressed in normal tissues and thus any aberrant ALK expression represents rearrangement in *ALK*. IHC has been used to detect *ALK* rearrangement in ALCL for many years using the ALK-1 antibody from Daiko. However, the promoter of EML4 is less active than the promoter for nucleophosmin that is fused to ALK in ALCL hence ALK-1 from Daiko is not a sensitive antibody for detecting *ALK* rearrangement in lung cancer [30]. Reverse transcription-polymerase chain reaction (RT-PCR) [10] and target deep sequencing [31] have also been used to detect *ALK* rearrangement in lung cancer. One of the major advantages of the sequencing approach is that the exact fusion variant is known [31] and that any unique clinicopathologic characteristics associated with each ALK fusion variant may be identified. Finally there are conflicting data on the prognostic significance of *ALK* rearrangement in lung cancer ranging the wide spectrum as a poor prognostic factor [32, 33] to no significance [34] to being a favorable prognostic factor [35]. In the future when next generation sequencing is in wide use then the biology of each unique ALK fusion will be even better identified and the prognostic significance of *ALK* rearrangement can be answered also. It is now believed that most of the *ALK*-rearranged lung cancer reminds dependent on ALK signaling for its pathogenesis as a majority of *ALK*-rearranged lung cancer patients continued to derive clinical benefit with continual ALK inhibition with an overall survival close to 30 months [36]. Hence *ALK* rearrangement in lung cancer is generally a favorable prognostic factor since currently there are several potent ALK inhibitors in clinical development and the motto of precision cancer medicine of “the right drug for the right disease” did certainly prolong life [34].

Treatment of *ALK*-Rearranged Lung Cancer

The discovery of *ALK* rearrangement in lung cancer would have been a historical academic footnote for awhile had it not been the existence of a phase I trial of crizotinib, a multi-targeted MET/ALK/ROS1 inhibitor [15, 16]. Crizotinib was initially being developed as a MET inhibitor [37] but its anti-ALK activity was known [38]. The discovery of a RTK rearrangement in a common epithelial malignancy is unexpected but the crizotinib phase I investigators and Pfizer were able to modify the protocol to accommodate this newly discovery cohort of molecularly defined patients. Within 2 years crizotinib was able to show impressive anti-tumor activity with overall response rate of approximately 60 % and progression-free survival of approximately 9 months essentially independent of the line of therapy [15, 16]. Based on the expanded phase I and a global phase 2 study of crizotinib received US FDA approval on August 26, 2011 for the treatment of advanced *ALK*-rearranged lung cancer [9]. Subsequently crizotinib has been demonstrated to be superior to chemotherapy in first-line (Pfizer Press release, March 25, 2014) or second-line treatment of *ALK*-rearranged lung cancer [22] in terms of statistically significant improved progress-free survival.

Invariably, these patients develop disease progression while on crizotinib. Many of these patients are likely to be still dependent on ALK signaling as they can benefit from continuation of crizotinib beyond treatment especially if the new and/or progressing metastatic site can be controlled by loco-ablative therapy [36, 39]. Recent retrospective analysis of data of crizotinib trials suggested progression free survival from the date of progression among patients who continued crizotinib beyond progressive disease (CBPD) was 16.4 months comparing to 3.9 months who discontinued crizotinib at progression (Hazard ratio [HR] 0.27, 95 % confidence interval [CI]: 0.17–0.42; $p<0.0001$) [36] and an overall survival of 29.6 months among CBPD patients comparing to an overall survival of 10.8 months among patients who discontinued crizotinib on progression (HR 0.30, 95 % CI: 0.19–0.46; $p<0.0001$) [36]. Furthermore the continual dependence of ALK signaling was the phenomenon of "disease flare" where there is rapid tumor progression when crizotinib is discontinued after the patient has progressed on crizotinib [40]. We can generally view there are two major mechanisms that result in disease progression on crizotinib. First close to 50 % of patients on crizotinib will develop progression in the brain (new or existing) likely indicating a pharmacodynamics failure of crizotinib to reach adequate therapeutic level in the central nervous system (CNS) [41]. Second the *ALK* gene acquired secondary resistance mutations to crizotinib including gatekeeper mutation (L1196M) and solvent front mutations (G1202R) [31, 42–46]. There are other resistance mechanisms reported such as activating of other by-pass signaling pathway such as EGFR [46] or the loss of ALK dependence altogether [43]. There are now several more potent ALK inhibitors in clinical development (ceritinib, alectinib, AP26113, ASP3026, X396, TSR-011, PF0663922, RXDX101) [47]. Among these ALK inhibitors, alectinib [48, 49] and ceritinib [50] are the farthest along in clinic development where phase I results of both compounds

have been published or presented and global phase 2 trial of both compounds in crizotinib-resistant patients have been completed. In general most of the ALK inhibitors can overcome most of the acquired resistance mutations except the solvent-front mutation G1202R [31, 51]. Thus an ideal ALK inhibitor for the treatment of *ALK*-rearranged lung cancer should have two major properties: the ability to penetrate CNS and have clinical activity in existing brain metastasis and the ability to overcome secondary acquired *ALK* mutations especially the solvent front mutation G1202R. However there are evidence both in vitro and from patient series that these ALK inhibitors have differential sensitivity to the various secondarily acquired mutation [13, 31, 52]. Thus rebiopsying crizotinib resistant tumor to try to understand exactly the resistance mechanism(s) will be necessary to tailor the treatment and serve as a paradigm for precision cancer medicine. Crizotinib and other ALK inhibitors in clinical development have put *ALK*-rearranged lung cancer in the parlance of precision oncology medicine. We also gained insight into the biology of *ALK*-rearranged lung cancer when we understand the mechanism of resistance to these inhibitors.

Future Perspective

One of the most commonly asked questions is what cause(s) *ALK* rearrangement in lung cancer. While no one has generated specific scientific data that can answer this one particular question, one can speculate knowing that there are RTK rearrangements are found in other common solid epithelial malignancies and the same fusion partners are found in different RTK [52], there may be chromosomal breakpoints in the cancer genome that is susceptible for intra- and interchromosomal rearrangement during DNA replication. Nevertheless the exact triggering event still eludes us. *ALK* rearrangement has been found in colon, breast, [52] and even thyroid cancer where one thyroid cancer patient with *EML4-ALK* rearrangement has responded to crizotinib [53]. Thus ALK signaling in lung cancer serves as a paradigm for future understanding of ALK signaling in other common epithelial malignancies and in broader perspective RTK rearrangement in epithelial malignancies [52].

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Chemotherapy Resistance in Lung Cancer

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Abstract Despite a growing interest in development of non-cytotoxic targeted agents, systemic chemotherapy is still the mainstay of treatment for both non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). However, chemotherapy resistance limits our ability to effectively treat advanced lung cancer. Some lung tumors are intrinsically resistant to chemotherapy, and in virtually all cases, even the initial responders rapidly develop acquired resistance. While targeting histology could result in enhanced tumor sensitivity to a particular chemotherapeutic agent, better understanding of molecular determinants of chemotherapy sensitivity/resistance would be critically important. Development of predictive biomarkers to personalize chemotherapeutic agents and combining novel agents targeting specific resistance pathways with standard chemotherapy could be some promising strategies to overcome chemotherapy resistance in lung cancer. In this chapter, we will discuss some key mechanisms of resistance for commonly used chemotherapeutic agents in lung cancer.

Keywords Chemotherapy resistance • Drug resistance • Lung cancer • Non-small cell lung cancer • Small cell lung cancer • Multidrug resistance

Platinum Drugs

Despite such a popularity of targeted agents across various solid tumors, targeted agents have rarely if at all cured any metastatic solid tumors. However, the truth is that platinum-based chemotherapy, also the main treatment regimen for advanced lung cancer, has cured many patients with metastatic testicular cancer [1]. There is a lack of understanding as to why testicular cancer cells but not other types of solid tumors such as lung cancer are exquisitely sensitive to platinum drugs.

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Despite the multifactorial nature of platinum resistance, reduced intracellular drug accumulation is one of the most consistently identified features of cisplatin-resistant cell lines [2, 3]. Reduced uptake of cisplatin has also been demonstrated in resistant NSCLC cells [4–6]. To clinically validate reduced drug accumulation as a significant mechanism of platinum resistance in NSCLC, a recent study established correlation between intratumoral tissue platinum concentrations and tumor response in NSCLC [7]. A significant relationship between tissue platinum concentrations and percent reduction in tumor size was demonstrated. The same relationship was seen individually in cisplatin and carboplatin groups. Patients with lower platinum concentration also had shorter time to recurrence, progression-free survival (PFS) and overall survival (OS) [7]. Furthermore, there was no significant impact of potential variables such as the type of platinum compound, number of cycles, cumulative dose and time lapse from last chemotherapy, which strongly suggests that certain resistance-inducing transport factors, are likely modulating intratumoral platinum accumulation.

CTR1 regulates uptake of copper which is a vital micronutrient for eukaryotic development and plays a significant role in platinum uptake. Deletion of the *Ctr1* gene in yeast and murine cells resulted in reduced accumulation of cisplatin and increased cisplatin resistance [8]. Conversely, enhanced uptake of carboplatin and oxaliplatin was seen when *Ctr1* gene was transfected into small cell lung cancer cell lines, supporting the importance of CTR1 in uptake of various platinum drugs [9]. In NSCLC patients, low expression of CTR1 was associated with poor prognosis in response to platinum-based therapy [10]. Given promising preclinical studies, there is a great potential for developing CTR1 into a therapeutic target. CTR1 and thus platinum uptake could potentially be manipulated using copper chelating drugs [11], the DNA demethylating agent decitabine [12] and a novel platinum-complex that does not rely on CTR1 for uptake.

Na+, K+ ATPase is also associated with increased intracellular accumulation and efficacy of cisplatin in lung cancer cell lines [13–15]. Inhibition of thromboxane A2 which antagonizes Na+, K+ ATPase increased cisplatin uptake and cytotoxicity by upregulating interleukin-1β-converting enzyme which is reduced in some platinum-resistant NSCLC cells [15, 16]. Furthermore, the glucose metabolite sorbitol decreased cisplatin cytotoxicity, Na+, K+−ATPase activity, and cisplatin uptake, suggesting a possible mechanism for cisplatin resistance in poorly controlled diabetes [17]. Tumor retention of Thallium-201 (T201) on SPECT scanning may reflect Na+, K+ ATPase activity. In a study involving SCLC patients who were treated with cisplatin-based chemotherapy, tumor response was associated with pre-treatment T201 retention [18], but this was not reproducible in an independent study in which patients with SCLC received a variety of chemotherapeutic agents [19]. Therefore, the role of T201 scanning in lung cancer is unclear.

There are also platinum export transporters such as the copper transporter ATP7B. Even though ATP7B mRNA and IHC expression significantly correlated with cisplatin resistance in NSCLC xenografts [20], ATP7B expression determined by IHC in clinical specimens did not correlate with PFS or OS following platinum-based chemotherapy [10]. It remains uncertain if ATP7B plays a role in

chemotherapy resistance in lung cancer. Breast cancer resistance protein (BCRP) is another drug efflux transporter (also known as ABCG2) that may be implicated in platinum resistance. Tumor IHC expression of BCRP was significantly associated with lack of tumor response and shortened survival in NSCLC patients undergoing platinum-based chemotherapy [21–23]. Furthermore, blood BCRP concentrations were significantly higher in chemo-resistant patients than in chemo-sensitive patients [24]. These findings support a possible role for BCRP in lung cancer chemotherapy resistance.

Drug detoxification may also contribute to intratumoral platinum concentration and platinum resistance in lung cancer. Glutathione (GSH) may bind cisplatin, thereby decreasing formation of platinum-DNA adducts. It may also contribute to increased repair of platinum-DNA adducts [25]. Several studies involving NSCLC and SCLC cell lines suggest that increase in GSH content was associated with decreased platinum-DNA binding [4, 26, 27] and intracellular platinum accumulation [28] which corresponded to increase in cisplatin resistance [4, 5, 26–32]. Conversely, factors that reduced cellular GSH increased sensitivity to cisplatin [4, 33] (Fig. 1 and Table 1).

Once platinum drugs enter the cell, they must form DNA adducts, which induce a cascade of signaling transduction pathways that lead to activation of p53-dependent and p53-independent cell death [34]. In NSCLC patients who were treated with concurrent cisplatin/XRT, low cisplatin-DNA-adduct staining in buccal cells was associated with worse survival [35]. While DNA adduct level is a

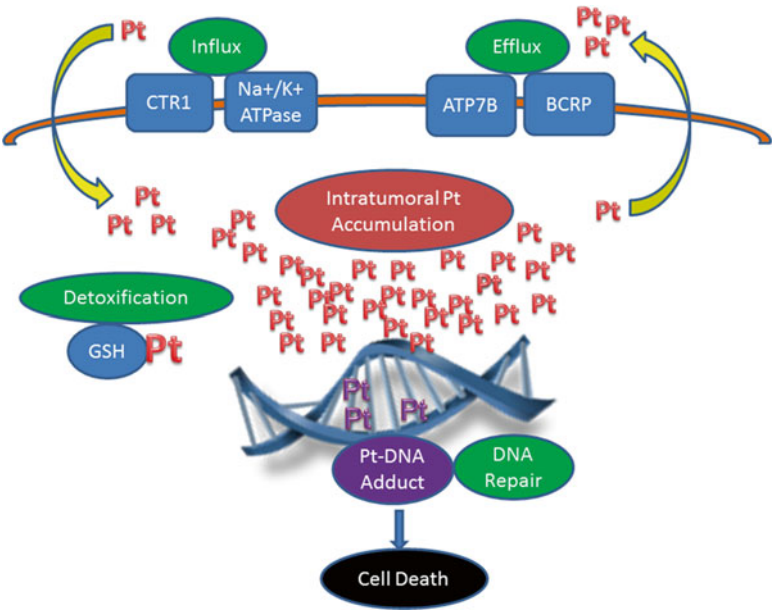

Fig. 1 A simplified overview of platinum resistance in lung cancer. *Pt* platinum, *CTR1* copper transporter 1, *GSH* glutathione, *BCRP* breast cancer resistance protein

Table 1 Tumor factors contributing to resistance to commonly used chemotherapeutic agents in lung cancer

Agents	Factors
Platinum	Drug accumulation Copper transporters (CTR1, ATP7B) Na+, K+ ATPase Breast cancer resistance protein Drug detoxification Glutathione DNA repair Nucleotide excision repair (ERCC1) Metallothioneins
Taxanes	Class III β-tubulin Expression levels Mutations Hypoxia inducible factor Histone deacetylase 6 Mitotic spindle checkpoint
Etoposide	Drug uptake Topoisomerase II alpha Rad51 Mitogen-activated protein kinases (MAPKs) ERK1, ERK2 Metallothionein Non-homologous end-joining repair
Gemcitabine	Human equilibrative nucleoside transporter 1 (hENT-1) Ribonucleotide reductase M1 and M2 (RRM1 and RRM2)
Vinorelbine	Polo-like kinases (PLKs) Class III β-tubulin Delta2 α-tubulin RLIP76 Stathmin (oncoprotein 18) Mitotic spindle checkpoint
Pemetrexed	Drug transport Proton-coupled folate receptor Folate receptor-α

[a]There are also multidrug resistance proteins such as the multidrug resistance protein (MRP) family or the multidrug resistance (MDR) P-glycoprotein family which could promote resistance to multiple chemotherapeutic agents

critical determinant of cisplatin cytotoxicity, increased DNA adduct repair decreases the apoptotic process [34]. Nucleotide excision repair is the major pathway for platinum adduct repair. The excision repair cross-complementation group 1 (ERCC1) protein is involved in nucleotide excision repair pathway and its expression correlated with cisplatin resistance in NSCLC [36]. There was a major interest in further developing ERCC1 as a potential biomarker for platinum sensitivity in NSCLC when Olaussen and colleagues demonstrated that ERCC1-negative NSCLC specimens determined by IHC correlated with significantly

longer OS following adjuvant chemotherapy [37]. There have been several studies with conflicting results since then. For example, a phase III trial designed to determine the influence of ERCC1 mRNA on tumor response following chemotherapy failed to show a correlation between ERCC1 expression and treatment response [38]. Furthermore, Friboulet and colleagues failed to validate the predictive effect of ERCC1 protein expression by IHC in two independent phase III trials (the National Cancer Institute of Canada Clinical Trials Group JBR.10 and the Cancer and Leukemia Group B 9633 trial from the Lung Adjuvant Cisplatin Evaluation Biology project) [39]. The authors concluded that currently available ERCC1 antibodies do not have adequate discrimination for clinical decision making regarding cisplatin-containing treatment regimens in patients with NSCLC, which requires the specific detection of the unique functional isoform of ERCC1 [39]. Furthermore, the results from the randomized international phase III trial of ERCC1 and RRM1 expression-based chemotherapy versus gemcitabine/carboplatin demonstrated no significant difference between the experimental arm and the control arm in PFS or OS [40]. Therefore, utility of ERCC1 as a predictive marker for platinum-based chemotherapy appears to be limited at this moment.

Metallothioneins are low molecular weight proteins that are involved in zinc homeostasis and may also be involved in chemotherapy binding and detoxification. Increased metallothionein expression was noted in some cisplatin-resistant NSCLC [5] and SCLC [41] cell lines. In patients with SCLC receiving cisplatin-based therapy, metallothionein expression determined by IHC correlated significantly with short survival [42].

Taxanes

The taxanes such as paclitaxel and docetaxel stabilize microtubules inhibiting the disassembly process, resulting in cell death due to large numbers of spurious asters forming throughout the cytoplasm [43]. Taxane-resistant NSCLC cell lines had significantly increased expression of class III β-tubulin [44–46]. Furthermore, the expression level of endogenous hypoxia inducible factor (HIF)-1 alpha appears to modulate taxane sensitivity in vitro by influencing the conformation and dynamics of microtubules [47]. In NSCLC cell lines that are resistant to taxanes, there was increased microtubule instability [48]. In addition, histone deacetylase 6 (HDAC6) decreased microtubule stability and antagonized the effect of paclitaxel on NSCLC cells. On the other hand, the farnesyl transferase inhibitor lonafarnib blocked the effect of HDAC6 on tubulin and was synergistic with paclitaxel [49]. The specimens from NSCLC patients demonstrated that a majority of the tumor samples expressed class II and class III tubulins, although the percentage of positive cells varied significantly among tumors [50]. NSCLC patients whose tumors expressed low levels of class III beta-tubulin isotype had a better response rate, longer PFS, and OS. However, this variable was not predictive in patients receiving regimens without tubulin-binding agents [51]. A multivariate analysis demonstrated that low-level

class III beta-tubulin expression was independently associated with PFS and OS, suggesting that the expression levels of class III beta-tubulin in tumor cells are predictive of response to therapy and patient outcome in patients with NSCLC receiving paclitaxel-based chemotherapy [51].

In addition to the expression levels of beta-tubulin, the mutations in beta-tubulin gene may also predict response to the taxanes. Beta-tubulin mutations in exons 1 or 4 were found in 33 % of NSCLC patients, and none of these patients had an objective response to paclitaxel treatment [52]. In the same study, median survival was 3 months for the patients with beta-tubulin mutations and 10 months for the patients without mutations [52]. Overall, the available data suggest that elevated expression and mutations in class III β-tubulin may predict resistance to taxanes in lung cancer, offering an opportunity for personalization.

Another potential mechanism of taxane resistance in lung cancer is through dysregulation of the mitotic spindle checkpoint. The mitotic spindle checkpoint, which blocks segregation of abnormal chromosomes, is often defective in human lung cancer cell lines [53]. Anti-microtubule agents such as taxanes activate the mitotic spindle checkpoint [53]. In NSCLC cell lines, impairment of the mitotic spindle checkpoint was associated with marked reduction in the ability of docetaxel to induce apoptosis, compared to cell lines with an intact mitotic spindle checkpoint [53].

Etoposide

Etoposide is an inhibitor of topoisomerase II enzyme that prevents re-ligation of the DNA strands leading to potentially lethal DNA breakage [54]. There is relatively little information available on the potential role of decreased drug uptake in etoposide resistance, although etoposide uptake is significantly higher in sensitive SCLC cell lines than in more resistant NSCLC lines [55]. The nonionic detergent Tween-80 increased etoposide uptake and cytotoxicity in NSCLC cells [56]. On the other hand, some etoposide-resistant cell lines have significantly increased level of cholesterol [57], which is thought to increase cell membrane rigidity.

As expected, SCLC cell lines are generally more sensitive to topoisomerase II inhibitors than NSCLC cell lines are [55, 58]. Kasahara and colleagues reported that nuclear topoisomerase II activity was twofold higher in SCLC cells than in NSCLC cells [55]. On a similar note, the expression of topoisomerase II-α mRNA and the protein levels in lung cancer cell lines were lower in the resistant variants compared to the sensitive variants [59]. Other etoposide-resistant SCLC cells derived from a patient who developed acquired resistance after initial response to etoposide demonstrated reduced topoisomerase II unknotting activity and reduced topoisomerase II-α expression [60]. In clinical tumor specimens, there was a significantly greater topoisomerase II-α expression by IHC in SCLC than in NSCLC [61, 62], and topoisomerase II-α IHC expression decreased significantly in SCLC tumors after therapy with etoposide [62]. Overall, these data suggest that reduced expression of topoisomerase II-α may increase resistance to etoposide.

Rad51 protein expression may play a role in etoposide resistance. In SCLC cells, resistance to etoposide correlated with protein levels of RAD51. Also, aberrant RAD51 gene expression altered both the efficacy of etoposide and repair of etoposide-induced DNA breaks [63]. Rad51 was expressed in 41 % of clinical NSCLC tumor samples but it did not correlate with resistance to etoposide [36]. Hence, the role of Rad51 in etoposide resistance will need further investigation in clinical setting.

ERK1 and ERK2 are examples of mitogen-activated protein kinases (MAPKs), and are downstream from Ras, Raf and MEK in the Ras pathway. Some etoposide-resistant SCLC cell lines had markedly increased MAPK activity [64]. Etoposide is less effective in hypoxic tumor cells [65]. A hypoxic environment activated the ERK pathway and increased resistance of NSCLC cells to etoposide. Conversely, inhibiting the ERK pathway reversed the hypoxia-induced resistance [65].

Other potential mechanisms for etoposide resistance in lung cancer include increased metallothionein expression and non-homologous end-joining (NEJH) repair. In addition to increased metallothionein expression seen in some cisplatin-resistant cell lines as discussed in Sect. 1, metallothionein expression also correlated with resistance to etoposide [66]. Exposure of lung cancer cell lines to cadmium or zinc increased metallothionein synthesis and increased resistance to etoposide [66]. NHEJ is a pathway that repairs double-strand breaks in DNA without the need for a homologous template. DNA-dependent protein kinase (DNA-PK) plays a crucial role in this pathway [67]. The efficacy of etoposide and etoposide-induced double strand breaks in SCLC cell lines varied with expression of DNA-PK proteins, suggesting a role for NHEJ repair in etoposide resistance [63].

Gemcitabine

Gemcitabine is a deoxycytidine analogue that, following uptake through nucleoside transporters, undergoes complex intracellular conversion to gemcitabine diphosphate and triphosphate that are important for cytotoxic effects [68]. Human equilibrative nucleoside transporter 1 (hENT-1) plays a role in cellular uptake, and hENT-1-deficient cells were demonstrated to be resistant to gemcitabine [69]. Liposome encapsulation may enhance gemcitabine uptake and cytotoxicity [70]. One NSCLC study reported that pretreatment hENT-1 expression did not directly correlate with tumor response or survival following gemcitabine-based chemotherapy, but only 16 % of the tumor specimens expressed hENT-1 by IHC [69]. In a separate study, none of the NSCLC patients who lacked hENT-1 expression determined by IHC responded to gemcitabine-based therapy [71].

Ribonucleotide reductase M1 (RRM1) encodes the regulatory subunit for ribonucleotide reductase which is a target of gemcitabine [72]. Ribonucleotide reductase plays an important role in cell growth and DNA repair [72]. Over-expression of RRM1 mRNA [72, 73] and genetic variations in RRM1 gene [74] correlated with resistance to gemcitabine in NSCLC cells. Bexarotene which counteracts RRM1

gene amplification [75] and RRM1 siRNA [76] both decreased resistance to gemcitabine. Furthermore, a significantly higher RRM1 mRNA expression was found in SCLC cell lines compared to NSCLC cells [77]. In clinical setting, NSCLC patients who were treated with gemcitabine alone or in combination with a platinum drug, RRM1 mRNA expression levels inversely correlated with tumor response [72, 78], time to progression [79] or survival [78–81]. Furthermore, RRM2, a related factor, also correlated inversely with response. Patients whose tumors had high expression of both RRM1 and RRM2 had significantly lower response rates, shorter time to progression and OS, compared to those whose tumors had low expression of both [82]. RRM1 expression appears to be a promising biomarker for gemcitabine-based therapy in NSCLC. However, a recent phase III study comparing ERCC1 and RRM1 expression-based chemotherapy with gemcitabine/carboplatin in advanced NSCLC demonstrated that there was no statistically significant difference in PFS and OS between the experimental arm and the control arm [40]. Further investigation is warranted.

Vinorelbine

Vinorelbine is an amphiphilic Vinca alkaloid with superior activity in NSCLC compared with other drugs in the same category [83]. It exerts its antitumor activity by binding to tubulin and inhibiting microtubule assembly, thereby preventing cell mitosis [84].

Stuckler and colleagues reported a potential role of RLIP76, a non-ATP binding transport protein, in facilitating the efflux of vinorelbine in NSCLC [83]. They demonstrated that RLIP76 catalyzes the transport of vinorelbine in a saturable manner that is dependent on vinorelbine and ATP [83]. Furthermore, threefold overexpression of RLIP76 in NSCLC and SCLC confers increased resistance to vinorelbine by decreasing intracellular vinorelbine concentration [83].

Polo-like kinases (PLKs) play a role in mitotic entry, spindle pole function and cytokinesis [85]. Expression of PLK 1 was elevated in NSCLC, and inhibiting it disrupted microtubule polymerization while potentiating the efficacy of vinorelbine [86]. NSCLC patients who had low class III β-tubulin mRNA expression in their tumors had significantly longer time to progression and OS than patients with high expression of class III β-tubulin following treatment with vinorelbine/cisplatin [79, 87]. Low Delta2 α-tubulin expression was also associated with significantly longer OS in advanced NSCLC patients treated with cisplatin/vinorelbine [87]. Conversely, when NSCLC patients received cisplatin/vinorelbine in adjuvant setting after undergoing surgical resection of their tumors, a greater benefit of the therapy was seen in patients with high versus low class III β-tubulin. Furthermore, the adjuvant therapy appeared to overcome the negative prognostic effect of high β-tubulin [88]. It is unclear why there is a discrepancy between adjuvant and metastatic setting in the influence of tubulin expression on vinorelbine efficacy.

Stathmin (oncoprotein 18) is a protein that plays an important regulatory role in tubulin dynamics. Transfection of the gene into lung cancer cells increased sensitivity to vinca alkaloids [89]. However, in patients with advanced NSCLC treated with vinorelbine plus cisplatin, time to progression was shorter in patients with high stathmin than with low stathmin mRNA expression [79]. Hence, its role in resistance remains unclear.

Similar to docetaxel as discussed in section "Taxanes", impairment of the mitotic spindle checkpoint was also associated with significant reduction in the ability of vinorelbine to induce apoptosis, compared to cell lines with an intact mitotic spindle checkpoint [53].

Pemetrexed

Pemetrexed is one of the newer agents that is commonly used to treat advanced non-squamous NSCLC based on a phase III study by Scagliotti and colleagues that showed survival differences based on histologic type [90]. In this study, OS was superior for pemetrexed/cisplatin versus gemcitabine/cisplatin in patients with non-squamous NSCLC only [90]. Like all other chemotherapeutic agents, virtually all tumors develop resistance to pemetrexed.

The proton-coupled folate receptor [91], the reduced folate carrier [91, 92] and the folate receptor-α [93] all appear to play a role in transport of pemetrexed. As discussed above, pemetrexed is more active in non-squamous carcinomas than in squamous cell carcinomas [90], possibly related to the fact that adenocarcinomas in particular have significantly higher expression of folate receptor-α compared to squamous cell carcinomas [94]. Cytotoxicity due to pemetrexed occurs by inhibiting thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT). Increased expression of these enzymes was associated with reduced cytotoxicity of pemetrexed in NSCLC cell lines [95]. Moreover, a recent meta-analysis evaluated the predictive value of TS and reported that NSCLC patients with lower TS expression could potentially benefit from pemetrexed-based chemotherapy [96]. High extracellular folate pools also markedly reduced pemetrexed cytotoxicity [97]. Further clinical investigation is warranted.

Multidrug Resistance Protein (ABCC, ABCB)

The factors that confer resistance to one agent may render tumors resistant to several other agents [98]. Alternating multiple agents with different mechanisms of action including cisplatin and paclitaxel does not improve clinical outcome in NSCLC [99]. The multidrug resistance (MDR) phenotype can be caused by transporters of the multidrug resistance protein (MRP) family (also known as ABCC) or MDR P-glycoprotein family (also known as ABCB).

A high proportion of SCLC [100, 101] and NSCLC [100, 102] cell lines express MRP mRNA, with greater MRP protein and/or mRNA expression in NSCLC than in SCLC cell lines [103, 104]. MRP expression is associated with decreased cellular drug accumulation of cisplatin [105], paclitaxel [106] and other agents [107]. In both SCLC and NSCLC cell lines, MRP mRNA or protein expression correlated significantly with resistance to vinca alkaloids [102, 103, 105, 107, 108], etoposide [100, 103, 104, 108], docetaxel [109], paclitaxel [106], gemcitabine [76] and cisplatin [103, 105]. However, an association between MRP expression and resistance was not seen in some cell lines [110–112]. In clinical specimens, MRP mRNA and/or protein by IHC was found in 32–100 % of NSCLC tumor specimens [100, 113–115]. MRP expression was significantly higher in more differentiated tumors than in less differentiated tumors [113] and in squamous cell carcinomas than in other NSCLCs [115]. MRP expression was also reported in SCLC clinical tumor samples [101].

The clinical value of tumor MRP expression remains uncertain. Response rates to platinum-based combinations were significantly lower in patients with SCLC whose tumors express MRP1 [116, 117] or MRP2 [118] compared to those with tumors that do not express these factors. Furthermore, MRP1 expression in SCLC tumors was significantly higher at relapse after treatment with cisplatin/etoposide compared to untreated tumors [116], suggesting that chemotherapy may upregulate expression of MRP. Likewise, in autopsy NSCLC tumor specimens, mRNA expression levels of MRP3 [119] and MRP5 [120] were significantly higher in patients who had been exposed to platinum drugs *ante mortem* than in patients who had not received platinum agents.

In advanced NSCLC, response rates to cisplatin/irinotecan were higher and survival was longer in patients with some MRP2 host genotypes than with other genotypes [121]. However, there was lack of correlation between MRP IHC expression and response to platinum-based combinations in other studies [21, 122]. In a different study involving NSCLC patients, MRP mRNA expression only correlated negatively with tumor response only in adenocarcinomas, and not in squamous cell carcinomas [123]. Furthermore, tumor MRP1 or MRP2 IHC expression did not correlate with survival in patients with resected NSCLC receiving adjuvant cisplatin plus a vinca alkaloid or etoposide [124]. Overall, preclinical data support a role for MRP in resistance to several types of chemotherapy. However, clinical data remain inconclusive. Further study is necessary.

Like MRP, MDR/P-glycoprotein may also render tumors resistant to chemotherapy by transporting drugs out of cells. In NSCLC cells, increased MDR1 mRNA and/or protein expression levels were associated with resistance to vinca alkaloids [102, 125–128], etoposide [125, 127], and taxanes [125–127, 129, 130]. MDR1/P--glycoprotein expression did not correlate significantly with sensitivity to platinum drugs [5, 110, 125, 126] or intracellular platinum accumulation [5, 110]. Some NSCLC and SCLC cell lines transfected with the MDR1 gene had augmented sensitivity to gemcitabine, and this augmented sensitivity was reversed by the P-glycoprotein inhibitor verapamil [112]. MDR1 gene overexpression was also seen in SCLC cell lines selected for resistance by exposure to paclitaxel [106] or

etoposide [131, 132]. P-glycoprotein expression correlated with HIF-1 alpha expression in NSCLC cell lines [133] and in resected NSCLC tumors [134]. Its expression was higher in lung adenocarcinoma cells under hypoxia [133], but was reduced in tumors of patients who had nitroglycerin patches applied to improve tumor blood flow and oxygenation prior to surgical resection [134].

In clinical setting, MDR1 mRNA and/or P-glycoprotein were expressed in 11–32 % of chemo-naïve specimens [62, 135–138], but were expressed in 61 % of tumors that had been treated with chemotherapy [137]. However, MDR1 expression in NSCLC did not correlate with histology or with clinical characteristics [135]. In SCLC, MDR1 expression was seen in 13–60 % of tumor biopsy samples [62, 101, 138].

With MDR1/P-glycoprotein, there is stronger evidence of an association of expression with clinical outcome in SCLC than in NSCLC. In SCLC, there was a negative correlation between expression of P-glycoprotein and clinical outcome in patients treated with cisplatin-etoposide [116, 117, 138–141]. In addition, P-glycoprotein expression was significantly increased in tumors previously exposed to therapy compared to the expression in untreated tumors [62, 116]. Efficacy of cisplatin/etoposide in SCLC patients also correlated with MDR1 host polymorphisms, with a significantly better chemotherapy response in patients with the 3435 CC genotype (exon 26) compared with those who had both 3435 CT and TT genotypes [142].

In NSCLC, there was a significant correlation of tumor P-glycoprotein IHC expression with response in two studies using platinum and paclitaxel [143, 144]. Similar to the findings in SCLC, the MDR1 3435 CC host genotype was associated also in NSCLC with a better response to cisplatin-vinorelbine compared with the combined 3435 CT and TT genotypes [145]. However, in a number of other studies involving patients with advanced NSCLC [21, 138, 146], P-glycoprotein expression by IHC did not correlate with response to cisplatin-based regimens [21, 138, 146] that included vinca alkaloids [21, 138, 147], taxanes [21] or gemcitabine [21]. Furthermore, host MDR1 C3435T polymorphisms did not correlate with outcome in NSCLC patients who were treated with cisplatin/docetaxel [148].

P-glycoprotein antagonists have been assessed in both NSCLC and in SCLC. The P-glycoprotein antagonist verapamil enhanced paclitaxel accumulation and vinorelbine efficacy in P-glycoprotein overexpressing lung cancer cells [111]. The epidermal growth factor receptor (EGFR) inhibitor gefitinib also reversed P-glycoprotein mediated taxane resistance in NSCLC cell lines [130, 149], but did not translate to improvement in efficacy of chemotherapy in randomized clinical trials involving NSCLC patients [150, 151]. A phase II study investigating cyclosporine A, another P-glycoprotein antagonist, in combination with paclitaxel in NSCLC patients suggested a possible positive impact of the cyclosporine on paclitaxel efficacy [152]. However, when cyclosporine was added to etoposide plus cisplatin in treatment-naive NSCLC, there was no evidence of clinical benefit [153]. Similarly, while hydroxyurea is thought to reverse *MDR1*-associated resistance in vitro [154], combining paclitaxel with hydroxyurea in previously-treated patients with advanced NSCLC did not appear to improve clinical outcome [155].

Based on the above, there is strong preclinical evidence of an association between MDR1/P-glycoprotein expression and resistance to several agents in lung cancer cells, but the clinical evidence is inconclusive for an association with outcome especially in NSCLC.

Conclusion

Despite rapid advances in diagnostic technology and better understanding of lung tumorigenesis which have led to development of targeted agents, chemotherapy continues to remain as the backbone of treatment for both SCLC and NSCLC. There are several chemotherapeutic agents with different mechanisms of action being currently used to treat lung cancer. However, virtually all tumors develop resistance to all these agents. Persistent investigation to understand the molecular mechanisms of chemotherapy resistance and identification of predictive biomarkers for chemotherapy sensitivity are necessary. Improved understanding of resistance mechanism at the molecular level may offer opportunities to combine chemotherapeutic agents with molecularly targeted agents, which may be a promising strategy to overcome chemotherapy resistance and to optimize therapy for lung cancer patients.

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Current State of Metal-Based Drugs for the Efficient Therapy of Lung Cancers and Lung Metastases

Bernhard Biersack and Rainer Schobert

Abstract Lung cancer is the second most common cancer in both men and women and thus a leading cause of cancer-related deaths worldwide. New efficient treatments especially for its advanced stages and metastases are desperately needed, particularly with regard to overcoming the resistance which thwarts the efficacy of most clinically established drugs such as the platinum complexes. Glimpses of hope are new metal-based drugs that have emerged over the past decade which displayed efficacy in patients with platinum-resistant tumors and metastases. This chapter provides an overview of the latest developments of such metal-based drugs against lung cancer.

Keywords Platinum complexes • Ruthenium complexes • Gadolinium • Ferrocene derivatives • Anticancer agents • Lung cancer • Lung metastasis • Multi-drug resistance • Tumor targeting

Introduction

More than one million people die of lung cancer worldwide every year. While non-smokers are almost exclusively affected by non-small cell lung cancer (NSCLC), small-cell lung cancer (SCLC) is characteristic of smoking patients [1, 2]. The platinum complex cisplatin is widely applied for the chemotherapy of a number of solid tumors including lung cancers. Second generation complexes like carboplatin and oxaliplatin entail less pronounced side effects and show significant activity in cisplatin-resistant cancer types [3]. These platinum(II) complexes kill cancer cells by modification of DNA and subsequent induction of apoptosis [4]. Today, cisplatin and carboplatin play a major role in the treatment of

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small-cell and non-small-cell lung cancers, customarily administered in combination with other drugs like gemcitabine, etoposide, vinorelbine or paclitaxel [1, 2]. The survival of patients could be improved significantly since the introduction of the platinum complexes into lung cancer therapy about 30 years ago. In 2004 the receptor tyrosine kinase inhibitor erlotinib was approved for the treatment of advanced and metastatic NSCLC which leads to an average extension of the survival time of about 3.3 months.

The success of platinum complexes has also intensified the development of anticancer drugs derived from metals other then platinum, e.g., ruthenium. Ruthenium complexes broadened the spectrum of antitumoral effects since many of them are much less toxic than platinum drugs while selectively targeting tumor metastases. Some ruthenium complexes (e.g., NAMI-A, KP1019) already entered clinical trials and showed pronounced efficacy in lung and colon cancer patients [5].

In the following, an overview is presented of various metal–based anticancer agents including platinum, ruthenium, gadolinium and iron compounds at an advanced stage of preclinical evaluation or in clinical trials for the targeted treatment of lung cancer and lung metastases with an emphasis on those types associated with a poor prognosis.

Platinum Complexes

Cisplatin and carboplatin are approved worldwide for the treatment of lung cancer diseases in combination with other drugs. But there are more platinums which are approved locally in East Asia, namely in China and in Japan, for the treatment of lung cancer. Nedaplatin (**1**), diammine[hydroxyacetato(2−)-*O*,*O′*]platinum(II) (Fig. 1), is another second generation cisplatin analogue comparable with carboplatin [6, 7]. Nedaplatin was approved in Japan for the treatment of NSCLC and SCLC in 1995 at doses of up to 90 mg/m^2 [8]. Dose limiting toxicities are thrombocytopenia and neutropenia [8]. A recent study using nedaplatin with irinotecan followed by gefitinib revealed an overall response of 43 % [9]. The glycolate leaving group of nedaplatin hydrolyses more rapidly than the carboplatin oxo-ligand cyclobutane-1,1-dicarboxylate giving the complex a reactivity comparable with that of cisplatin [6]. However, nedaplatin shows only slight interaction with serum proteins. Hence, nedaplatin is less nephrotoxic than cisplatin and carboplatin and is recommended for the treatment of patients with impaired renal functions instead of cisplatin. As expected, the DNA lesions caused by nedaplatin are comparable with those inflicted by cisplatin since the spectator ligands are the same (ammine ligands). Lobaplatin (**2**, Fig. 1) was developed in Germany (Astra Medica AG, Frankfurt) and was approved in China for the treatment of small-cell lung cancer (SCLC). It is given as a diastereomeric mixture of complexes derived from racemic lactic acid and shows no nephro-, neuro-, and ototoxicity [10–14]. In addition to these approved examples of platinum complexes against lung cancer diseases there are further Pt-based drugs that entered clinical trials. Zeniplatin

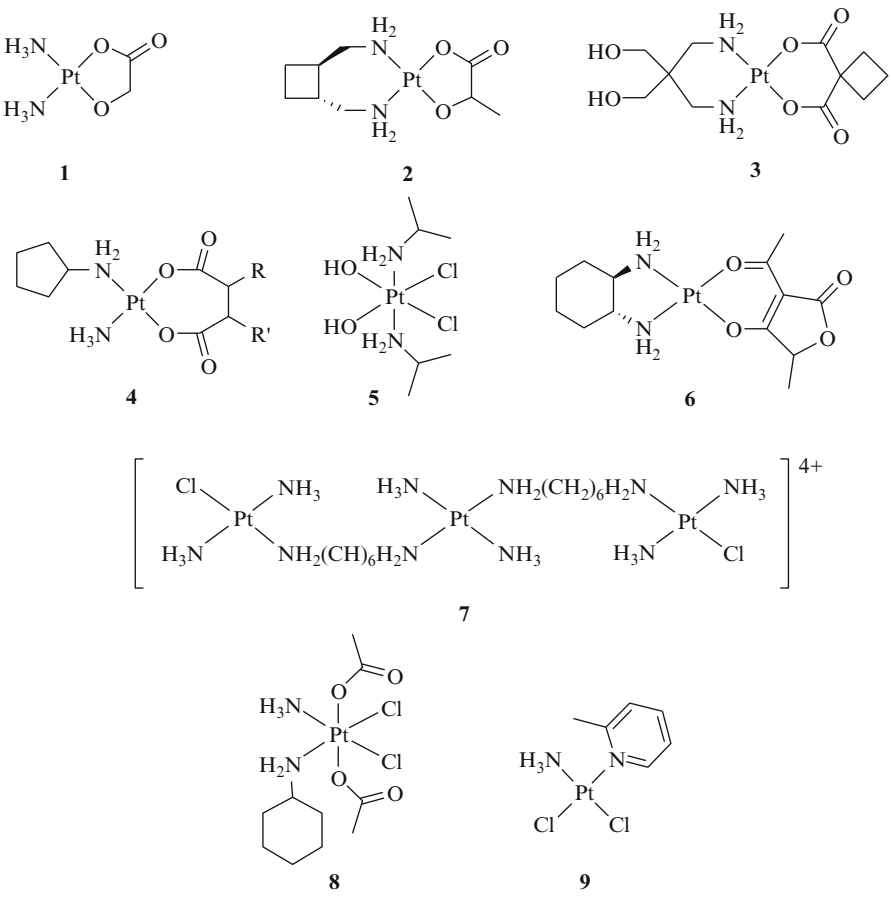


Fig. 1 Platinum complexes which entered clinical trials

(**3**), for instance, shows higher water solubility than cisplatin due to the additional hydroxyl groups (Fig. 1). It underwent phase II trials for advanced NSCLC patients but provoked only moderate response (10–14 %) [15]. Zeniplatin was eventually abandoned due to serious nephrotoxicity which had gone unnoticed during the phase I trials. Cycloplatam (**4**) was developed by Russian scientists and showed particularly high activity in lung cancer xenograft models (Fig. 1) [16]. Cycloplatam was investigated in a series of phase II trials in Russia against various solid tumor diseases, however, its development was stopped for unknown reasons. Iproplatin (**5**) is an octahedral platinum(IV) complex and actually a prodrug which undergoes reduction to cytotoxic Pt(II) complexes in vivo (activation by reduction) (Fig. 1) [17]. It underwent several phase II trials, also for NSCLC and SCLC, however, it could not surpass the activity of cisplatin in these trials and its development was discontinued [18, 19]. The complex TRK710 (**6**) features a

3-acyltetronate ligand coordinated to the Pt(II) center and showed higher activity and uptake rates in cisplatin-resistant cancer cells (Fig. 1). A distinct activity in NSCLC was confirmed and initial phase I clinical trials revealed lower nephrotoxicity and myelosuppression than cisplatin [20]. However, development was terminated for unknown reasons and there are no further reports of clinical trials with this interesting complex. BBR3463 (**7**) was the first non-classical platinum complex of clinical relevance (Fig. 1). This cationic trinuclear platinum complex consists of two functional *trans*-Pt moieties connected by a tetraamine-platinum bridge. This complex revealed an enhanced cellular uptake compared with cisplatin and caused a high degree of interstrand crosslinks in DNA which are not recognized by repair enzymes of the NER eventually leading to a p53-independent apoptosis induction [21, 22]. Although positive results were observed for NSCLC-patients of a phase II trial (2 OR and 11 PR from 33 patients) the drug never entered clinical phase III trials [23].

While most of the platinum drugs have to be administered intravenously, Satraplatin (**8**) was the first orally applicable platinum agent under clinical investigation (Fig. 1). Like iproplatin it is a Pt(IV) complex prodrug which is reduced in the organism to active Pt(II) complexes (activation by reduction) that build up DNA lesions similar to cisplatin [24]. Satraplatin when given alone rendered a partial response rate of 38 % in patients with SCLC. Clinical trials in combination with paclitaxel for patients with NSCLC are ongoing [25]. Picoplatin (**9**) is another orally applicable Pt(II) complex initially designed to meet the glutathione-mediated resistance towards platinum drugs (Fig. 1). The methyl group of the picoline ligand hinders the attack of deactivating bionucleophiles such as glutathione and metallothionein at the Pt(II) center, and an enhanced activity of picoplatin in resistant cancer cells was actually observed [26, 27].

Ruthenium Complexes

While primary solid tumors can be efficiently removed by surgery in many cases the emergence of metastases is frequently responsible for the lethal issue of cancer diseases. Hence, suitable treatments which prevent or reduce metastasis are in great demand. The promising ruthenium(III) based drug candidate NAMI-A ("New Antitumor Metastasis Inhibitor"), ImH[*trans*-Im(DMSO)$RuCl_4$] (Fig. 2) initially failed the common anticancer screens such as the NCI 60 cell lines screen yet prevented in additional animal studies the development and growth of lung metastases derived from lung and breast carcinomas [28–30]. The high efficacy of NAMI-A in the lungs is based on its eightfold longer half-life in lung tissue compared with other tissues and organs which is probably due to the high content of collagen which can interact with NAMI-A. In addition to its anti-invasive properties NAMI-A also possesses anti-angiogenic activity as to assays with blood vessels in the chorioallanthoic membrane (CAM) of chicken and in rabbit cornea probably by binding to NO released from the endothelial cells [31]. In contrast to platinum drugs NAMI-A

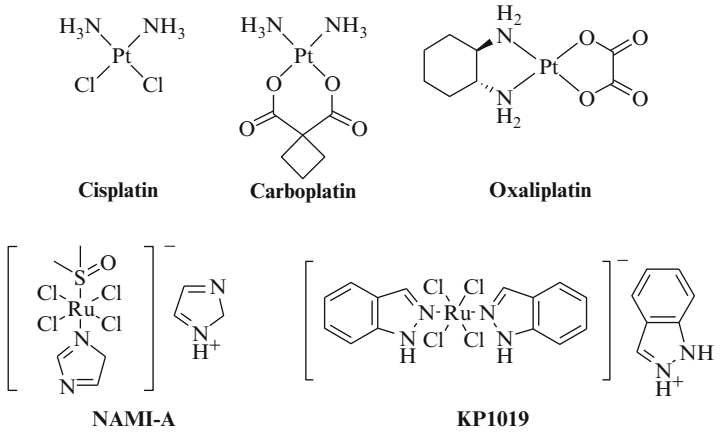


Fig. 2 Platinum and ruthenium complexes with proven anticancer activity

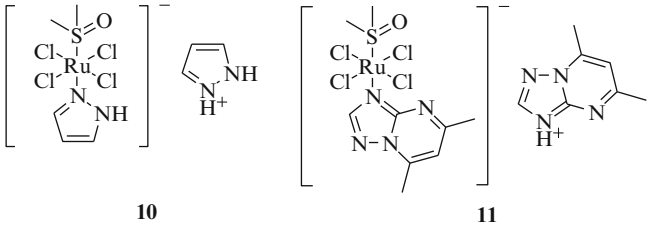


Fig. 3 NAMI-A-type Ru(III) complexes with high activity against lung cancer and lung metastases in lab animals

binds only weakly to DNA. NAMI-A inhibits the MAPK pathway by suppression of membrane PKC leading to apoptosis and it reduces cell migration as well as the release of gelatinase [32, 33]. A phase I study in 2004 revealed a high tolerance of the drug by patients with the appearance of blisters being the only dose-limiting toxic event [34].

Meanwhile NAMI-A type compounds with improved stability in aqueous solutions have emerged. Complex **10** (Fig. 3), for example, with a less basic pyrazole ligand distinctly reduced the number of lung metastases arising from Lewis lung carcinoma and MCa breast carcinoma [35]. In the lung cancer model **10** showed similar activity compared with NAMI-A while in the breast cancer model it was twice as active as NAMI-A with respect to the reduction of lung metastases. Thus, complex **10** behaves similarly to NAMI-A. However, it did not arrest the cell cycle at the G2/M checkpoint like NAMI-A. On the other hand, **10** inhibited tumor cell migration in matrigel more efficiently than NAMI-A. Another NAMI-like complex is (Hdmtp)

[*trans*-$RuCl_4$(DMSO)(dmtp)] (**11**) bearing a 5,7-dimethyl[1, 2, 4]triazolo[1,5-*a*] pyrimidine ligand (dmtp) (Fig. 3) [36]. Complex **11** exhibited antimetastatic properties comparable to those of NAMI-A, but also revealed a higher liver toxicity and caused edema. However, it showed a lower kidney toxicity than NAMI-A.

Organoruthenium complexes are usually more stable than NAMI-A-type coordination complexes concerning ligand exchange and were therefore identified as promising candidates for new anticancer drugs as well. RAPTA-C (**12**) is a ruthenium(II)-arene complex bearing a 1,3,5-triaza-7-phosphaadamantane (PTA) ligand (Fig. 4). The η^6-arene ligand (*p*-cymene) stabilizes the reactive Ru(II) state of the complex. Complex **12** showed pH dependent DNA damage, in particular at lower pH values which are typical of hypoxic tumor sites when the PTA ligand is protonated [37]. Despite of the structural differences between NAMI-A and RAPTA-C (**12**) their modes of action showed similarities. Like NAMI-A complex **12** is inactive against the primary tumor, yet highly efficacious against lung metastases. Treatment of MCa mammary carcinoma bearing CBA mice with 200 mg/kg/day of **12** for 2 days reduced the number of lung metastases by more than 50 % with two mice of the treated group turning entirely cancer free [38]. While the half-life of complex **12** is comparable with that of NAMI-A it showed enhanced distribution and blood clearance rates. Hence, complex **12** (RAPTA-C) appears a suitable candidate for further testing including clinical trials.

Sadler and co-workers prepared the ruthenium(II) complexes RM175 (**13**) and HC11 (**14**) which exhibit significant cytotoxic activity in cancer cells, including cisplatin-resistant cells (Fig. 4) [39]. These complexes coordinate selectively to the N7-site of guanine bases forming an additional hydrogen bond between an NH_2 group of the en ligand and the C6-carbonyl group of guanine. Both complexes were distinctly growth inhibitory in A549 lung non-small cell lung cancer xenografts at doses of 25 mg/kg. A significant liver toxicity was observed at higher doses for complex **14**. The authors suggest that liver toxicity is attributed to the arene ligand

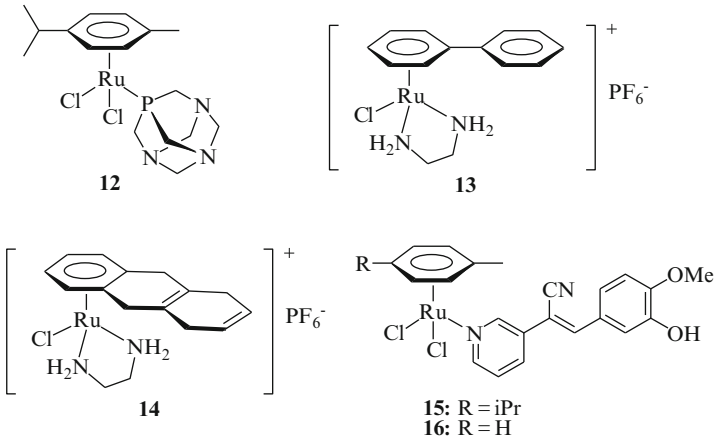
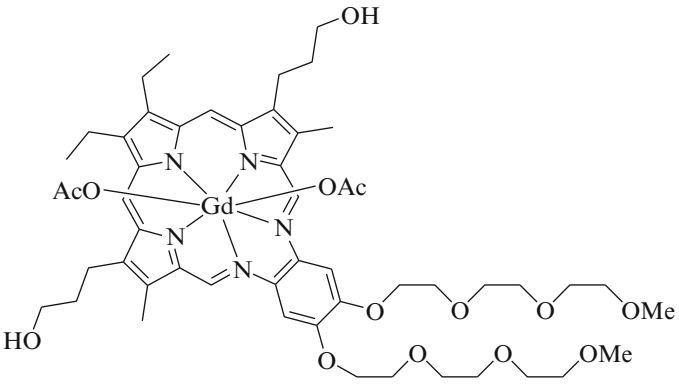


Fig. 4 Organometallic arene-Ru(II) complexes with potential against lung cancer

and further modification of the arene ligand is in progress in order to reduce the hepatotoxicity of such ruthenium complexes. Inspired by the successful launch of the EGFR-inhibitor erlotinib as a drug against advanced NSCLC, Biersack et al. have recently prepared neutral arene-Ru(II) complexes **15** and **16** of pyridine-based tyrphostine derivatives which are well known EGFR inhibitors (Fig. 4) [40, 41]. These complexes exhibited growth inhibitory activity in various cancer cell lines at sub-micromolar concentrations and led to strong DNA metallation in vitro.

Motexafin Gadolinium

Between 100,000 and 300,000 cancer patients are diagnosed with brain metastases in the US alone every year and their survival expectancy is dauntingly poor [42]. Since radiation therapy is the treatment of choice for brain metastases, radiosensitizing agents were developed in order to improve the impact of the radiation therapy and to reduce side effects. The MRI-detectable complex motexafin gadolinium (**17**, MGd), also known as gadolinium texaphyrin (Xcytrin, NSC 695238) is a metal-based anticancer agent that showed promising results as a radiosensitizer (Fig. 5). This agent features a Gd(III) ion chelated by a porphyrin-like texaphyrin ligand. Due to its high electron affinity complex **17** readily oxidises cellular components, e.g., reducing metabolites, leading to lethal DNA damage [43]. In addition, complex **17** directly targets enzymes like thioredoxin reductase and ribonucleotide reductase which are often overexpressed in cancer cells [44], and it interferes with intracellular zinc levels in cancer cells probably due to a direct oxidation of zinc metallothionein [45]. The pleiotropic anticancer effects of **17** led to various clinical trials, e.g., against NSCLC and brain metastases thereof. In these trials the performance of complex **17** is characterised by a high tumor-selectivity and tolerable side-effects and toxicities. In combination with whole-brain radiation therapy (WBRT) complex



17

Fig. 5 Motexafin gadolinium (**17**)

17 revealed reduced neurotoxicities and neurological progression as well as improved quality of life in patients with NSCLC-derived brain metastases in a phase III study (SMART) [46].

Ferrocene-Based Compounds

The discovery of ferrocene (**18**, Fig. 6) and the elucidation of its sandwich structure in the 1950s by G. Wilkinson (Imperial College London) and E. O. Fischer (Technical University Munich) was a milestone in the field of inorganic chemistry and was awarded with the Nobel Prize in 1973. The simple complex ferrocene activates lymphocytes and exerts distinct antitumor effects by redox-sensitive signaling involving oxidation of Ras proteins at cystein sites [47]. Recently, Kenny and coworkers prepared ferrocene conjugates bearing peptide fragments. Complex **19**, for instance, features a combination of a ferrocene component, a naphthoyl linker and a small peptide moiety which has shown excellent activity in the H1299 non-small cell lung cancer cell line (IC_{50} = 0.62 μM) and so exceeded the efficacy of cisplatin in these tumor cells by far (Fig. 6) [48, 49]. The naphthoyl linker of complex **19** lowers the oxidation potential of the conjugated ferrocene while the peptide fragment may form many hydrogen bonds to the biological targets. Snegur and coworkers prepared the new ferrocenylethyl benzotriazole **20** and studied it concerning its in vivo activity in lung cancer xenografts via the subrenal capsular assay (SCA) in comparison with cisplatin (Fig. 6) [50]. Complex **20** exhibited low toxicity and caused 45 % regression in non-small cell lung cancer models at doses of 18 mg/kg/day, while cisplatin led to merely 23 % regression in this tumor model. The same group found that complex **20** alkylates nucleobases like adenine forming ferrocenylalkyl adenine. This mild way of ferrocenylalkylation is assumed to be the

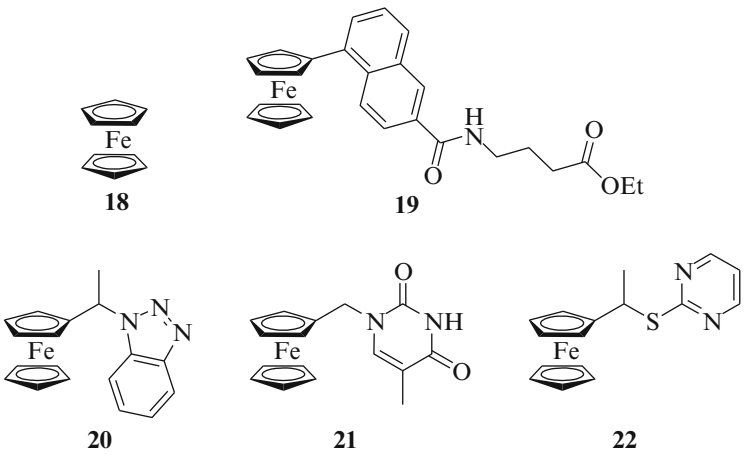


Fig. 6 Ferrocene-based compounds with activity against lung cancer

reason for the selective triggering of endonucleases at the early stage of apoptosis without causing necrotic effects. In continuation of this interesting discovery Simenel et al. have recently disclosed new ferrocene conjugates with nucleobases. Ferrocenylmethyl thymine **21** showed distinct in vivo activity in various solid tumors (Fig. 6) [51]. For instance, mice bearing Lewis lung carcinoma (LLC) when treated with **21** (5.0 mg/kg/day intraperitoneally) revealed a growth inhibition of the LLC tumor of 45 %. The combination of complex **21** (5.0 mg/kg/day) with the alkylating anticancer drug cyclophosphamide led to improved effects in the LLC xenografts (growth inhibition of 50 %). The maximum tolerated dose (MTD) for **21** was 20 mg/kg when given intraperitoneally. The same group also reported the preparation of a ferrocenylalkyl thiopyrimidine **22** (Fig. 6) with enhanced anticancer activity that can be given to mice at a higher dose than **21** [52]. LLC xenografts were treated with **22** (20 mg/kg/day) and showed a tumor growth inhibition of 65 %. The observed MTD for complex **22** was 150 mg/kg.

The compound class of retinoids comprises both natural vitamin A derivatives and synthetic analogs which are important for the differentiation of cells and the development of tissues and organs [53–55]. These effects are mediated by the binding of retinoids to nuclear receptors such as retinoic acid receptors (RARs) or retinoid X receptors (RXRs). All-*trans*-retinoic acid (ATRA) and bexarotene, for example, are currently applied for the treatment of various skin and cancer diseases. Their use is limited, though, by teratogenic and hepatotoxic side-effects [56–58]. Xiang and coworkers prepared new 13-*cis*-retinoyl ferrocenes in order to enhance the biological activity of retinoids against cancer [59]. Compound **23** (Fig. 7) exhibited improved activity in A549 lung cancer cells ($IC_{50} = 20.4$ μM) compared with 13-*cis*-retinoic acid ($IC_{50} = 35.8$ μM) alone. Jaouen and coworkers disclosed several ferrocene derivatives (so-called ferrocifens) of the selective estrogen receptor modulator (SERM) tamoxifen [60, 61]. A compound dubbed hydroxyferrocifen

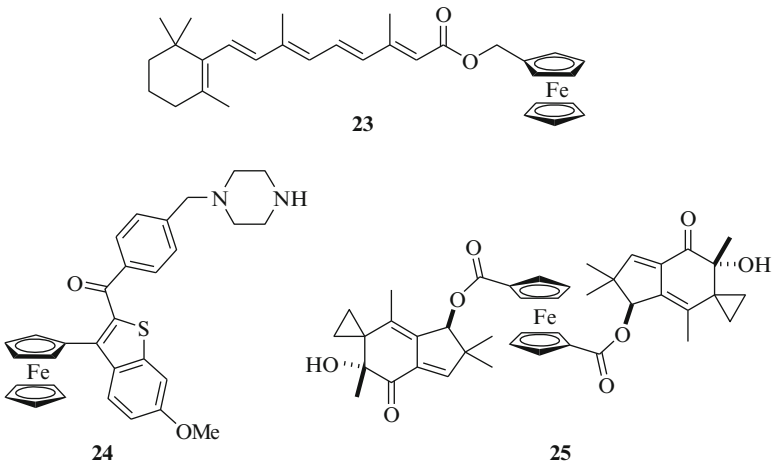


Fig. 7 Ferrocene conjugates with anticancer active compounds

exhibited distinct growth inhibitory activity in breast cancer cells, both ER-positive and ER-negative. Recently, Marques et al. disclosed a ferrocene-based raloxifen analogue **24** which also displayed some activity in lung cancer cells (Fig. 7) [62]. In A549 lung cancer cells complex **24** exhibited a distinctly better growth inhibition (IC_{50} = 3.85 μM) than cisplatin (IC_{50} = 18.13 μM). Generally, compound **24** showed no cross-resistance to cisplatin and is obviously no substrate for the ABC-transporters of multi-drug resistant cells.

Ferrocenes can also increase the selectivity of anticancer agents and shield reactive drugs sterically or electronically as observed in the case of the fungal cytotoxin illudin M. Illudins M and S and the semisynthetic compound irofulven are alkylating agents which are activated by reduction of their enone system either by NADPH-dependent oxido-reductases or by glutathione [63]. Irofulven was found to be efficacious against MV522 metastatic lung carcinoma xenografts and to increase the life expectancy of treated animals by more than 150 %, a value that exceeds the one of mitomycin C by far (increase in life span of 61 %) [64]. In addition, irofulven blocked the formation of lung metastases in an animal model resistant to classical anticancer drugs. However, irofulven remained inactive in a clinical phase II trial against advanced non-small cell lung cancer [65]. In a search for more selective illudin M derivatives, Schobert and coworkers developed ferrocene conjugates of illudin M [66, 67]. The ferrocene diester **25** (Fig. 7) was active at sub-micromolar IC_{50} values against various cancer cell lines while being forty times less toxic than illudin M in non-malignant fibroblast cells. The ferrocene-attached illudin M moieties of **25** are less prone to reductive detoxification by glutathione than free illudin M, and its antiproliferative activity depends on active JNK-signaling. First in vivo assays of **25** in lab mice revealed no toxicity at doses of 25 mg/kg and further tests in suitable lung cancer xenografts are planned.

Conclusions

The platinum complexes cisplatin and carboplatin have been a mainstay in the therapy of lung cancer diseases right from the beginning. More platinum complexes were put through their paces in clinical trials and a good deal of them was found active against drug resistant and advanced lung cancers. In addition to platinum compounds, ruthenium and gadolinium complexes have emerged that selectively target metastases in the lung or lung cancer metastases in the brain. The drug candidates NAMI-A and motexafin gadolinium have already reached advanced stages of clinical trials and are likely to obtain approval for the treatment of lung cancer metastases. Organometallic sandwich complexes such as ferrocene pose a second line of development in the field of anticancer metallodrugs. When attached to intrinsically bioactive fragments complex conjugates with pronounced activity in lung cancer models may result. Ferrocenes in particular figure prominently among such conjugates, presumably due to their interference with reactive oxygen species and with detoxification of drugs.

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