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HHS Public Access

Author manuscript

Nat Rev Cancer. Author manuscript; available in PMC 2017 December 04.

Published in final edited form as:

Nat Rev Cancer. 2014 August ; 14(8): 535–546. doi:10.1038/nrc3775.

Non-small-cell lung cancers: a heterogeneous set of diseases

Zhao Chen[1,*], **Christine M. Fillmore**[2,3,4,*], **Peter S. Hammerman**[1], **Carla F. Kim**[2,3,4], and **Kwok-Kin Wong**[1,5,6]

[1]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts 02115, USA

[2]Stem Cell Program, Boston Children's Hospital, Boston, Massachusetts 02115, USA

[3]Harvard Stem Cell Institute, Cambridge, Massachusetts 02138, USA

[4]Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115, USA

[5]Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, USA

[6]Belfer Institute for Applied Cancer Science, Dana-Farber Cancer Institute, Boston, Massachusetts 02115, USA

Abstract

Non-small-cell lung cancers (NSCLCs), the most common lung cancers, are known to have diverse pathological features. During the past decade, in-depth analyses of lung cancer genomes and signalling pathways have further defined NSCLCs as a group of distinct diseases with genetic and cellular heterogeneity. Consequently, an impressive list of potential therapeutic targets was unveiled, drastically altering the clinical evaluation and treatment of patients. Many targeted therapies have been developed with compelling clinical proofs of concept; however, treatment responses are typically short-lived. Further studies of the tumour microenvironment have uncovered new possible avenues to control this deadly disease, including immunotherapy.

Lung cancer results in the largest number of cancer-related deaths worldwide[1,2]. More than 85% of those cases are currently classified as non-small-cell lung cancer (NSCLC), for which the predicted 5-year survival rate is 15.9% — a figure that has only marginally improved during the past few decades[3]. Technological advances during the past decade, including the introduction of next-generation sequencing (NGS), the generation of multiple genetically engineered mouse models (GEMMs) of lung cancer and the construction of large databases characterizing the molecular features of human tumours, have transformed our view of NSCLC from histopathological descriptions to precise molecular and genetic identities that can be resolved to the single-cell level. In parallel, approaches and concepts from fields such as developmental biology, stem cell biology and immunology have deepened our knowledge of tumour development, cellular heterogeneity and interactions between the lung tumour and its surrounding microenvironment. These multidisciplinary

Correspondence to P.S.H., C.F.K. and K.-K.W. phammerman@partners.org; carla.kim@childrens.harvard.edu; kwong1@partners.org.
*These authors contributed equally to this work.

Competing interests statement

The authors declare no competing interests.

efforts have enhanced our understanding of molecular disease mechanisms, thereby forming the rationales for targeting different cellular compartments simultaneously. Scientists and physicians have better tools than ever to pursue answers to two provocative questions: first, how can we define the specific subsets of NSCLC that differ by cellular and molecular composition? Second, how can we effectively control lung cancer growth for each specific subset of NSCLC? In this Review, we discuss how data that are derived from technological advances in lung cancer genomics, mouse modelling of cancers and tumour microenvironment studies might be used to improve the survival of patients with NSCLC through the development of novel therapeutic strategies.

Defining NSCLC subsets

NSCLC is currently defined by pathological characteristics. The two predominant NSCLC histological phenotypes are adenocarcinoma (ADC; ~50%) and squamous cell carcinoma (SCC; ~40%)[4,5]. In general, ADCs arise in more distal airways, whereas SCCs arise in more proximal airways and are more strongly associated with smoking and chronic inflammation than ADCs[4,5]. ADCs often have glandular histology and express biomarkers that are consistent with an origin in the distal lung, including thyroid transcription factor 1 (TTF1; also known as NKX2-1) and keratin 7 (KRT7)[4,5]. By contrast, SCCs are characterized by squamous differentiation, which is more reminiscent of the pseudostratified columnar epithelium that lines the trachea and upper airways[4,6]. SCCs are distinguished from ADCs in the clinic by immunostaining for cytokeratin 5 and cyto keratin 6 and/or the transcription factors SRY-box 2 (SOX2) and p63 (REFS 4,5,7). Other subtypes of NSCLC include large cell carcinoma, which is diagnosed by exclusion if tumour cells do not appear glandular or squamous in shape or express ADC or SCC biomarkers, although it is unclear whether large cell carcinomas are genetically distinct from ADC or SCC[4]. Some neuroendocrine tumours are also classified as NSCLC, although the most aggressive form of neuroendocrine tumour is classified as small-cell lung cancer (SCLC)[4].

Genetic mutations and genomic heterogeneity

Although histological features and marker expression remain the basis of clinical tumour diagnosis, recent advances in NGS and other high-throughput genomic profiling platforms have allowed researchers to examine the breadth of genetic mutations within lung tumours. Following the identification of *KRAS* and *BRAF* mutations[8,9], epidermal growth factor receptor (*EGFR*) mutations were discovered in patients with lung ADC and were associated with response to EGFR inhibitors[10–13]. Further recurrent mutations and amplifications in many potentially targetable oncogenes have since been identified in lung ADC, including *HER2* (also known as *ERBB2*), *MET*, fibroblast growth factor receptor 1 (*FGFR1*) and *FGFR2*, as well as fusion oncogenes involving anaplastic lymphoma kinase (*ALK*), the *ROS1* receptor tyrosine kinase, neuregulin 1 (*NRG1*), neurotrophic tyrosine kinase receptor type 1 (*NTRK1*) and *RET*[14–22]. These oncogenic changes, many of which predict sensitivity to clinical inhibitors, jointly account for most cases of lung ADC[23–25]. For lung SCC, the number of tumours for which whole-exome sequencing is available is lower than for ADC but, so far, potentially targetable mutations in ADC do not seem to be prevalent in this histological subtype[20]. Instead, genes such as discoidin domain-containing receptor 2

(*DDR2*), *FGFR1, FGFR2, FGFR3* and genes in the PI3K pathway seem to be more commonly mutated in lung SCC[20]. Many of these mutations (with the exception of those in the PI3K pathway) have been validated by preclinical studies as driver mutations[22,26,27].

NGS studies have also revealed the molecular taxonomy of lung cancer and have shown a dazzling complexity of somatic alterations in NSCLCs that extends far beyond protein kinases to include epigenome modifiers, transcription factors, splicing factors and genes involved in cellular immunity[20,28,29]. Potentially important mutations and copy number gains identified from patient tumours are summarized in TABLE 1, with relevant preclinical and clinical evidence. Among the 21 different tumour types for which exome sequences were directly compared, lung SCC and ADC ranked second and third highest in median somatic mutation frequency, with an average of ten mutations per megabase of coding DNA sequenced[30]. It is worth noting that ADCs in non-smokers have 5–6-times fewer mutations[24,31]. Given this relatively large number of mutations per tumour, there will probably be more important mutations identified for NSCLC as the number of tumours that are analysed increases. An important challenge that remains is understanding which of these many mutations are important in lung carcinogenesis and/or treatment response, in contrast to those mutations that are merely a consequence of the tumorigenic process. Thus, the genomic profiles highlight the heterogeneity of the NSCLC genome and provide a plausible explanation for the highly heterogeneous treatment responses that we have observed in the clinic. By cataloguing a large collection of mutations for each patient, a more accurate evaluation of the net effects of genotype and therapy response may be achieved and will ultimately inform the most suitable treatment strategies.

Other novel technologies have also facilitated the discovery and validation of somatic mutations in lung cancer. For example, high-throughput screens using established short hairpin RNA (shRNA) libraries have identified genes that cause synthetic lethality with common oncogenic events, such as KRAS-activating mutations or p53 inactivation, leading to potential new treatment targets, such as TANK-binding kinase 1 (TBK1)[32]. Similarly, the application of mass spectrometry to metabolomic, proteomic and phosphokinase profiling, as well as single cell time-of-flight mass cytometry (cyTOF), have led to numerous new findings, including the discovery of recurrent aberrations such as the *ROS1* fusions and the potential diagnostic or prognostic marker isocitrate dehydrogenase 1 (IDH1)[17,33,34]. Such advances in high-throughput technology are promoting rapid advances in our understanding of NSCLC biology and, ultimately, will help to determine how NSCLC develops, spreads and can be better treated.

Heterogeneity in lung tumour microenvironments

The concept of tumour heterogeneity applies not only to tumour epithelial cells but also to the diverse microenvironments with which the tumour cells interact[35]. Carcinoma cells, in the lung and other organs, are closely associated with the extracellular matrix (ECM), mesenchymal cells such as fibroblasts, infiltrating immune cells and vasculature (FIG. 1). In some cases, this environment is essential to tumour initiation or tumour growth, whereas in other cases it can prevent tumorigenesis or even promote tumour clearance[35,36].

In lung tumorigenesis, genesis of new blood and lymphatic vessels supplies necessary nutrients for tumour growth and allows for an influx of immune cells of the myeloid and lymphoid lineages. The myeloid cells that are implicated in this process include tumour-associated macrophages (TAMs) and tumour-associated neutrophils[37]. Mice that harboured germline knock-in of kinase-dead inhibitor of nuclear factor-κB (NF-κB) kinase subunit-α (IKKα) developed spontaneous lung SCC that is characterized by NF-κB activation and marked accumulation of TAMs that were essential for disease progression[38]. Secretion of pro-angiogenic factors such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) by TAMs in lung cancer suggests why these cells are associated with increased microvessel formation[39,40]. Likewise, increased neutrophil numbers have been associated with poor prognosis in NSCLCs, perhaps owing to their ability to degrade matrices with elastase[41,42]. Neutrophils that are found in mouse tumours are phenotypically characterized as polymorphonuclear CD11B- and lymphocyte antigen 6G-expressing ($CD11B^+Ly6G^+$) cells, and are often considered to be a subtype of `myeloid-derived suppressor cells` (MDSCs)[43]. In the tumour microenvironment, accumulated MDSCs are thought to promote tumour progression by increasing matrix degradation, tumour cell proliferation, metastasis and angiogenesis[35,37].

Tumours can evade immune surveillance by expressing molecules that maintain tolerance to normal peripheral tissues, including the interaction of the tumour-associated programmed cell death 1 ligand 1 (PDL1) with the immune receptor programmed cell death 1 (PD1; also known as PDCD1). Recently, the use of antibodies targeting the PD1–PDL1 checkpoint has resulted in some marked responses in early-stage clinical trials for a large panel of therapy-refractory cancer subtypes, including advanced melanoma, NSCLC and renal cell cancer, with a proportion of responding patients showing persistent long-term benefits[44,45]. The PD1–PDL1 interaction inhibits $CD8^+$ cytotoxic T lymphocyte proliferation, survival and effector function, and can induce apoptosis of tumour-infiltrating T cells; PD1–PDL1 interactions can also promote the differentiation of $CD4^+$ T cells into forkhead box P3-expressing ($FOXP3^+$) regulatory T (T_{Reg}) cells, which are known to further suppress the immune system and cause peripheral immune tolerance in patients with lung cancer[46]. Despite the promising clinical benefits, there is currently no defined subset of patients with lung cancer who are particularly sensitive to PD1–PDL1 blockade. This is partly due to a lack in the understanding of how tumour cells affect their microenvironment, including the surrounding immune cells[44,45,47]. Evaluating the expression of PDL1 on tumour cells is only the starting point in the analysis of the interactions between tumour cells and the surrounding microenvironment[48,49]. Many important questions remain, including whether lung tumours with distinct genetic backgrounds differ in how they shape their immune microenvironment.

Differences in the ability to secrete inflammatory cytokines such as interleukin-6 (IL-6) may be one way in which tumour cells influence their surroundings[50,51]. Tumours that are driven by different oncogenic mutations in mice, such as *EGFR* and *Kras*, have distinguishable immune infiltrates with respect to cell types and their phenotypes in the tumour immune microenvironment[48,49]. These mechanisms have not been defined in detail, and there are other important questions to consider: does each genetic subset of NSCLC have its own

unique microenvironmental influences, or can common characteristics of how the surroundings drive tumour subsets be uncovered? How does targeted therapy alter the tumour microenvironment? Do drug-resistant or recurrent tumours have an environmental milieu that is distinct from the initial untreated tumour? A more thorough understanding of these dynamic interactions will help to show new targets that can be manipulated to promote antitumor effects. Importantly, many of these questions are challenging to understand, given the need to study the immune system *in vivo*, and the use of mouse models with intact immune systems in combination with patient samples may be instructive.

Cell(s) of origin for NSCLC heterogeneity at tumour initiation

Another contributing factor to the diversity of NSCLCs may be the potential distinct cells of origin in which subsets of NSCLC first arise. The cell of origin for each subset of NSCLC is essentially unknown beyond initial work in this area in mouse models. For example, it remains to be understood whether multiple cell types are equally likely to produce *KRAS*-mutant ADC or if only one cell type exists in the right microenvironment and must gain oncogenic *KRAS* expression to produce this type of ADC. It is possible that the biology of different cells of origin is what drives the different phenotypes of NSCLCs with distinct genotypes. This could be the result of unique gene expression patterns of the originating cells, differences in the type of cells that the originating cells can produce, or unique microenvironments of the originating cell type. Ultimately, the clinical importance behind these seemingly basic biological questions is whether a different cell of origin partly dictates treatment responses. Can knowledge of the cell of origin predict environmental influences that can be targeted for antitumour therapy? Furthermore, can knowledge of the cell of origin be used for the earlier detection of tumours? The answers to these questions have the capacity to revolutionize our current concept of the stratification, diagnosis and treatment of NSCLC.

A long-standing hypothesis proposes that stem and progenitor cells in adult tissues function as carcinoma cells of origin because they are the only cells that have a sufficient lifespan to accumulate the many genetic alterations required for tumour progression[52]. Furthermore, stem cells have inherent self-renewal capacity and may not need extensive epigenetic reprogramming. However, even genetically normal cells with limited self-renewal capacity can be induced to acquire more stem cell-like properties in response to genetic alterations or microenvironmental changes[53,54], and this supports the idea that more mature, differentiated cells may be just as likely to give rise to malignancy. Historically, ADCs have been proposed to arise from club cells (previously known as Clara cells) or alveolar epithelial type 2 (AT2) cells, owing to the staining of patient ADCs by immunohisto chemistry with markers of these cell types[4,5]. However, it is important to note that the staining pattern of a tumour is merely a snapshot of the gene expression of the tumour cells at that time point and might not match the initiating cell type. Our current understanding of cells of origin for lung cancer is mostly derived from experimental data using GEMMs[55] (BOX 1; FIG. 2). Many conditional GEMMs target activation and/or loss of genes specifically to lung cells by intranasal or intratracheal instillation of adenovirus-Cre, which infects lung epithelial cells along the proximal to distal tract. After using intra nasal adenovirus-Cre to induce oncogenic *Kras*, loss of *Pten* or loss of *p38*, the first hyperproliferative cells to be observed were

bronchioalveolar stem cells (BASCs) — implicating them as possible ADC cells of origin[56–58]. However, in more recent studies that targeted the expression of oncogenic *Kras*^{G12D} only in cells expressing club cell secretory protein (CCSP), such as club cells and BASCs, or only in cells expressing surfactant protein C (SPC), such as AT2 cells and BASCs, AT2 cells seemed to be the only cells that were capable of giving rise to advanced ADC in the alveolar space, whereas club cells and BASCs seemed to be limited to driving bronchiolar hyperplasia within the same time frame[59]. Using these approaches, it is notable that changes in the cells of origin were evident when the genotype for tumour initiation was altered (for example, to include p53 loss) or if injury or inflammation were present during tumour initiation (for example, after adenovirus infection or after naphthalene-induced injury)[56,59–63]. Injury or inflammation probably more closely mimics the scenario of tumour initiation in humans, in which environmental influences and ongoing injury occur in contrast to the relatively sterile mouse colony. These questions are unexplored in other models of ADC that use distinct oncogenes or in SCC. Thus, it remains entirely possible that club cells, AT2 cells and BASCs are all possible initiators of lung ADC. Future development of more precise lineage-specific Cre drivers combined with approaches to study tumorigenesis in the context of injury and inflammation (situations that are more likely to mimic carcinogenesis in humans) will be needed to better determine the comparable ADC-initiating activity of these populations.

Although increasing amounts of genomic data show that distinct gene expression programmes and driver mutations distinguish ADC from SCC, it remains unclear whether these two tumour types arise from a common cell of origin or diverse cell types, including different lung stem or progenitor cells (BOX 2). Until recently, a paucity of GEMMs for SCC has precluded analysis of the cells of origin of this important NSCLC subtype. It has long been hypothesized that SCC arises from basal cells, as lung SCCs most frequently arise in the proximal lung[4], but also because they often express KRT5, SOX2 and p63, which are markers of the normal basal cell population[5–7,62,64]. GEMMs of ADC have been more widespread, mostly owing to the usefulness and availability of the conditional oncogenic *Kras* allele (which drives lung ADCs both independently and more rapidly in combination with *Trp53* loss) as well as early models using chemicals that induce RAS mutations to drive tumours. Although *KRAS* or *NRAS* mutations are present in up to 25% of ADCs, they are rarely detected in SCCs, and mouse modelling with these oncogenes seems to result predominantly in the development of ADC. Mutations that are common in samples from patients with SCC have only recently been catalogued, and this opens up new ideas about how to model SCC[20]. Kinase-dead IKKα knock-in mice develop spontaneous lung SCC, but because this mouse has a germline *Ikka* mutation, it is not clear which cells in the lung expanded into the squamous tumours[38]. Loss of the tumour suppressor liver kinase B1 (*Lkb1*; also known as *Stk11*) in the oncogenic *Kras*^{G12D} model produces a mixture of tumours, including ADC, SCC and large cell carcinoma[65]. Similarly, a mixture of ADC and SCC is found in mice after targeted deletion of *Pten* or transforming growth factor-β receptor 2 (*Tgfbr2*) in proximal cells with keratin-driven Cre alleles in the *Kras*^{G12D} background[66]. Expression of the transcription factor *Sox2* (overexpressed in 20–60% of human SCCs) in club cells and BASCs produces lung tumours that express the marker p63 but histologically resemble ADCs[7]. This intriguing finding suggests that distal lung epithelia

are unable to produce a fully squamous phenotype, despite the expression of an SCC transcription factor. In addition, the deletion of *Lkb1* and *Pten* in the lung via intranasal adenovirus-Cre was recently shown to give rise to fully penetrant lung SCCs[49]. The next important step will be to use lineage-restricted Cre alleles, such as the oestrogen-responsive Cre under control of the *Krt5* promoter (*Krt5*–Cre-ER), to determine which lung cells that are null for *Lkb1* and *Pten* are able to drive squamous disease.

Tumour-propagating cells (TPCs) and cellular plasticity: heterogeneity between tumour cells

'Cancer stem cells' or TPCs, which are defined as the tumour cells with the stem cell-properties of self-renewal and differentiation, have the capacity to produce tumours in transplantation assays. Establishment of tumours at metastatic sites and tumour recurrence following treatment have been attributed to growth and survival of TPCs[67,68]. Recent studies have identified potential cell surface markers or genetic traits that may mark the TPC population in NSCLC, such as aldehyde dehydrogenase (ALDH) activity or expression of NOTCH, CD24, CD166 or CD44 (REFS 38,69–73). However, these studies have not used serial transplant assays in the context of the lung environment, and a *bona fide* human lung TPC remains to be defined. GEMMs have allowed for more systematic study of lung TPC phenotypes, including serially transplanted tumours and metastases. Studies in the $Kras^{G12D}$-expressing and *Trp53*-null model of ADC suggest that stem cell antigen 1 (SCA1, also known as Ly6A)$^{+}$, CD24^{+}, β4 integrin^{+}, and NOTCH3^{hi} mark the TPC population[70,73]. The identity of TPCs from other ADC GEMMs is unknown; SCA1 did not enrich for TPCs from the *Kras*- or *EGFR*-driven GEMMs[74]. In the first lung-specific genetic model of SCC (the *Lkb1*-and *Pten*-null model) the TPCs had a high expression of SCA1 and the basal cell marker nerve growth factor receptor (NGFR). Intriguingly, these TPCs also expressed high levels of the immune-checkpoint molecule PDL1, which may be targetable as described above[49]. Overall, these findings indicate the importance of taking the genotype of the tumour into account when seeking to define a TPC population; each subset of NSCLC might harbour TPCs with unique surface markers and molecular drivers, which could each be uniquely targeted. Alternatively, many subsets of NSCLC might not have one TPC population that can be defined. Future research focusing on resolving the metastatic activity and therapy response of murine TPCs and the molecules that control them may help to translate these findings to improve the treatment of patients with lung cancer.

The genetic complexity and rapid clonal evolution of lung tumours could mean that if TPCs do occur in most lung cancers, they will have a high degree of plasticity. Fascinating clinical observations have shown some patients who are initially diagnosed with EGFR-driven ADCs develop SCLC after long-term treatment with the EGFR tyrosine kinase inhibitors gefitinib or erlotinib[75,76]. In contrast to ADC models, lineage tracing and viruses that are engineered to express Cre under the control of various cell-type-specific promoters have been used to show that SCLCs probably arise from neuroendocrine cells[76–78]. However, examination of these tumours before and after SCLC conversion shows the persistence of the same *EGFR* mutations, suggesting a shared clonal origin of both types of tumours. This highlights the potential epigenetic plasticity of lung cells and lung tumours after therapy[75]. Further careful evaluation of TPC activity and cellular plasticity of tumour cells with patient

tissues, probably using patient-derived xenograft (PDX) models and GEMMs of lung cancer, will help us to better understand tumour lineage conversion as a path towards developing chronic treatment resistance. These findings also highlight the importance of considering how cells of origin may differ, depending on the therapeutic status of the tumour environment.

Integrated therapies for NSCLC

Target validation and patient stratification

Although studies of lung cancer genomes have implicated several genes as likely crucial mediators of tumour initiation and progression, experimental validation of the most important, functional genomic changes in lung cancer cells remains a challenge. Despite computational approaches to separate 'driver' alterations from passenger alterations, this distinction is probably more nuanced, and substantial work will need to be completed to model the consequences of specific genome alterations in NSCLC. Existing repositories of lung cancer cell lines, as well as efforts to generate new cell lines from patient tumours have led to a number of important discoveries, although these cell lines still fail to represent the full diversity of human NSCLCs[79]. Three-dimensional culture techniques might also offer a new way to propagate normal and tumorigenic lung cells to better probe vulnerabilities of tumour cells[49,73]. Multiple *in vivo* models using mice to recapitulate lung cancer disease processes and treatment responses have been generated, including GEMMs harbouring specific genetic aberrations that have been identified in human tumours[55,80] (BOX 1). Translation of the experimental results obtained through *in vitro* and *in vivo* modelling systems has formed the basis for current and future patient stratification paradigms (BOX 3). The limitations of these approaches should also be considered in future work to develop a more precise understanding of how to predict therapy response.

Current treatments for NSCLC

The past decade has seen some truly impressive new treatments for subsets of patients with lung cancer, most of whom harbour mutations in one of the key oncogenic driver mutants upon which tumour survival and progression are dependent. These include mutations in *EGFR*, the echinoderm microtubule-associated protein-like 4 (*EML4*)–*ALK* fusion and *ROS1* fusions[81,82]. Extensive preclinical and clinical studies have proven the marked treatment responses and survival advantages over conventional chemotherapies that are provided by target-specific inhibitors to EGFR-activating mutations or to ALK fusions[83–85]. Recent genomic analyses of lung SCC have also given the first set of potentially targetable driver mutations, including FGFR1, FGFR2, FGFR3, DDR2 and PI3K[20]. Clinical trials that aim to target these subsets of patients who have Stage I–IIIA lung cancers are currently underway; preliminary results were presented at the 2014 American Association for Cancer Research Annual Meeting[86], and these showed responses to an FGFR inhibitor (BGJ398) in a subset of patients with SCC who have *FGFR1* amplification.

Unfortunately, acquired resistance to chronic treatment often develops within 9–12 months in most patients who are treated with kinase inhibitors[84,87,88]. Therefore, patients who have Stage I–IIIA tumours are still treated by surgical resection as a first-line treatment and

receive combination chemotherapy as a standard of care, with the use of targeted agents still considered to be experimental. For patients with advanced disease who have progressed on an inhibitor of EGFR or ALK, several recurrent secondary mutations have been identified, such as EGFR-T790M and additional kinase domain mutations in ALK[87,88]. Hence, finding treatments for tumours that are resistant to first-generation EGFR or ALK inhibitors has been a recent focus. Several newly developed inhibitors that either have more potency or are rationally designed to favourably target the mutated kinases, such as AZD9291 and CO-1686 for EGFR and LDK378 for EML4–ALK, have generated promising initial clinical results[89–91]. Discovery of the mechanisms that underlie acquired resistance in patients without additional mutations in the primary driver gene is also greatly facilitated by high-throughput analytical approaches. Amplifications of *ALK* and alternative pro-cancerous pathway activations were identified in *ALK* fusion-positive patients who have become resistant to the first-generation ALK inhibitor crizotinib[87]. In patients who are resistant to chronic EGFR inhibitor treatment, a wide range of resistance mechanisms has been reported. These include increased activities of additional kinases owing to *MET, HER2* or *ERK* amplification, additional mutation of *PIK3CA* (which encodes the PI3K p110α subunit) or overexpression of AXL kinase[14,92–95]. Enhanced NF-κB signalling activity was also implied as one possible resistance mechanism that is evident by an improved response and survival in patients with EGFR mutations who have an increased expression of the NF-κB inhibitor IκBα (also known as NFKBIA)[96]. In addition, a common *BIM* (also known as *BCL2L11*) polymorphism that results in changes in splicing and the deletion of the pro-apoptotic BCL-2-homology domain (BH3) was shown to potentially mediate intrinsic resistance to EGFR inhibitors[97], highlighting the complexity of possible resistance mechanisms. It is conceivable that the comprehensive acquisition of information on different aspects of tumour biology, such as genomic and kinase profiling in patients, will be crucial in the future to determine the best course of treatment following any new diagnosis or the development of acquired resistance.

Most patients with advanced stage NSCLC without targetable genomic alterations are still treated by conventional chemotherapies. Activating KRAS mutations were identified and verified long before the discovery of mutant EGFR. However, treatment choices for patients with KRAS-mutant lung cancer are still very limited. Current efforts to treat this subset of patients have been mostly focused on inhibiting common KRAS downstream signalling cascades. The RAF–MEK–ERK pathway, which is activated directly downstream of KRAS, has proven to be a valid target in both preclinical models and clinical trials[98–100]. However, the clinical benefits of MEK inhibitors, even in combination with other agents, are still somewhat moderate compared to those of target-specific inhibitors such as erlotinib for patients with activating EGFR mutations, and the use of MEK inhibitors is associated with additional complications and enhanced toxicity[100]. The available preclinical and clinical results present clear challenges to the common belief that therapies targeting one or a few specific alterations should have fewer side effects and lower toxicity compared to standard chemotherapies. Indeed, this is not entirely a surprise, as many of the targeted pathways for lung cancer treatment are also essential for normal tissue functions. The simultaneous inhibition of multiple signalling pathways can be deleterious to necessary normal cells. One possible remedy being explored is to optimize treatment schedules and improve targeting

efficiencies for single-pathway inhibition by improving inhibitor potency or linear inhibition of multiple targets within the pathway. Nonetheless, alternative treatment approaches with less toxicity and better responses are of immediate need. A few studies have more recently reported the rational design of KRAS inhibitors that target the cysteine residue of the common KRAS mutation G12C in lung cancer[101,102], and these are therefore similar to the second-generation EGFR inhibitors (such as WZ4002, AZD9291 and CO-1686) that target EGFR-T790M. *In vitro* studies of these KRAS inhibitors demonstrate a proof of concept[101,102]; however, the *in vivo* efficacy of these molecules still requires much more investigation.

Targeting multiple cellular compartments in lung cancer

Similar to *KRAS* mutations, many newly identified potential pro-cancerous changes, such as overexpression of the transcription factors SOX2 and MYC[103–105], present clear challenges to our current ideas about treatment approaches — in cases in which there is no clear druggable target, what can be done? Furthermore, the short-lived *in vivo* efficacy for most if not all existing small molecule inhibitors[87,106] also advocates more durable treatment approaches. On the basis of our current understanding, the more effective approach probably requires therapies that not only target tumour cells but also target other components of the tumour, such as tumour vasculature, tumour-associated fibroblasts and tumour-specific and/or non-specific immune cells. Besides the more recently studied PD1–PDL1 inhibitory pathway, other approaches that intervene with the immune system, such as antibodies against `cytotoxic T lymphocyte protein 4` (CTLA4; also known as CD152), CD73 or CD47, and more sophisticated cellular immune therapies, such as engineered T cell therapy using `chimeric antigen receptors` (CARs), are also under extensive scrutiny[107–110]. More importantly, ongoing efforts are seeking to discover the best combination approach that integrates immune therapy with other therapies. Angiogenesis has long been seen as a possible therapeutic window, with many novel therapeutic agents that have been developed or are being developed to clinically target this process, although the overall efficacy of anti-angiogenic agents has been modest in unselected patient cohorts[111]. Emerging evidence has suggested that the combination of immunotherapy and anti-angiogenic agents has potential synergistic effects[112,113], pointing to a new possible avenue to mutually enhance both treatments. In addition to providing key nutrients and oxygenated blood, tumour vasculature might have a role in supporting TPCs[35,114]. Similarly, stromal cells such as fibroblasts have been shown to provide additional signals that support tumour growth and survival, and they may therefore have major roles in primary and acquired treatment resistance[35,115]. Understanding how best to target these various aspects of the tumour microenvironment would require a high-throughput comparison of changes in the tumour microenvironment that occur upon single and combination treatments.

Targeted therapies might also be able to indirectly regulate tumour growth. Two prominent examples are drugs that target epigenetic enzymes and metabolic enzymes. Targeting epigenetic enzymes is expected to enable marked perturbation of gene expression within tumour cells to stop tumour growth. The recently developed bromodomain protein inhibitors have shown efficacy in numerous preclinical studies[116], including in lung cancer[117], and they are currently under evaluation in the clinic, including in the ongoing Phase I clinical

trials NCT01987362 and NCT01587703. Variations in expression as well as recurrent mutations were also reported for several histone- and DNA-modifying enzymes, including enhancer of zeste homologue 2 (EZH2), TET methyl cytosine dioxygenase 2 (TET2) and DNA methyltransferase 3A (DNMT3A) in all subtypes of NSCLC[28]. In a similar concept, altered metabolism is one of the key features of cancer cells. Anti-diabetic drugs, insulin-like growth factor 1 receptor (IGF1R) inhibitors and drugs that target glycolysis or lipid, nucleic acid and amino acid synthesis are currently being explored for anti tumour activities in NSCLC[118–121]. Targeting metabolism is certainly promising for cancer control, particularly when combined with other approaches. Recent studies have also highlighted connections between TET and IDH, which could have resulted in a `CpG island methylator phenotype` (CIMP) in a subset of lung cancer, and this 'connects the dots' between epigenetics and metabolism[122–124].

Conclusion

The quickened pace of discovery of mechanisms that underlie lung cancer development and possible treatments in the past decade present the opportunity to integrate information from multiple approaches for future lung cancer treatment. Large amounts of information about the identity of individual lung tumours are being collected. New and improved functional studies are needed to meet the pace of data set generation, and all of the aspects of tumour heterogeneity — genetic, cellular and epigenetic — need to be integrated to determine better approaches to make an impact in this devastating disease. We anticipate the future treatment scheme to be a genotype-dependent, carefully selected combination that would ensure an enhanced tumour immune reaction, inhibition of angiogenesis and blockade of interactions between tumour cells and stromal cells. Thus, we advocate 'integrated therapy', in contrast to the current concept of targeted therapy, as the future of effective NSCLC treatment.

Acknowledgments

The authors thank United Against Lung Cancer, Thoracic Foundation, Bonnie J Addario Lung Cancer Foundation, Claudia Adams Barr Program For Basic Cancer Research, grant numbers CA122794, CA166480, CA163896, CA154303, CA120964 CA140594.

Glossary

Myeloid-derived suppressor cells (MDSCs)
MDSCs encompass a heterogeneous population of myeloid cells, which share the ability to suppress T cells through the production of arginase and the expression of inducible nitric oxide synthase (iNOS)

Pseudostratified epithelium
This describes the epithelium of the trachea, which is truly a monolayer but appears to have some stratification due to the variable distances of the nuclei from the basal lamina

Patient-derived xenograft (PDX)
Primary tumour cells from fresh patient tumours that are propagated subcutaneously in immunocompromised mice

EGFR-T790M
The most common mutation (~50%) in the epidermal growth factor receptor (*EGFR*) gene that confers resistance to EGFR tyrosine kinase inhibitors such as erlotinib and gefitinib

Cytotoxic T lymphocyte protein 4
(CTLA4; also known as CD152). A surface receptor that transmits inhibitory signals to T cells.

CD73
A cell surface enzyme that generates extracellular adenosine, which inhibits T cell function

CD47
The receptor for thrombospondin 1 (TSP1). CD47 is highly expressed in many tumour cells

Chimeric antigen receptors
(CARs). Genetically engineered receptors that result in desired specificity (to tumour cells) in effector T cells

CpG island methylator phenotype
(CIMP). Reflects the genomic status that multiple CpG islands are methylated simultaneously, leading to epigenetic inactivation of different genes, including tumour suppressors

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Box 1

Mouse models

Genetically engineered mouse models (GEMMs) have enabled numerous studies of non-small-cell lung cancer (NSCLC) that would not be possible using patient samples or cancer cell lines: for example, preclinical or co-clinical trials of targeted therapies, the study of metastatic and transplanted disease and examination of tumour cells of origin[25,49,74,98,125,126]. Today, GEMMs for most of the common NSCLC driver mutations have been generated, including for *KRAS*, epidermal growth factor receptor (*EGFR*), and echinoderm microtubule-associated protein-like 4 (*EML4*)–anaplastic lymphoma kinase (*ALK*); and despite their lack of genetic complexity compared to human cancers, they have shown some remarkable similarities in pathological features and treatment responses to the human disease[98,125–127].

GEMMs are particularly informative when the net effects of several mutations need to be determined *in vivo*. For example, the conditional oncogenic $Kras^{G12D}$ mouse model has been used to elucidate the steps from early to late tumorigenesis, owing to the temporal control it affords[128], and it is easy to combine with mice bearing conditional null alleles for other genes of interest. For example, $Kras^{G12D}$ tumours only reach a full adenocarcinoma stage with a very long latency, but $Kras^{G12D}$-expressing and *Trp53*-null tumours are more advanced and show a decreased response to certain treatment strategies when compared to $Kras^{G12D}$ tumours[128,129]. Simultaneous inactivation of *Pten* and liver kinase B1 (*Lkb1*) in the lung produced only squamous cell carcinoma (SCC)[49], and this fits with the preclinical observations that PI3K and mTOR pathways are activated in most human lung SCC tumours[20,49,65]. Similar genetic breeding schemes can be used to identify and validate potential treatment targets through *in vivo* synthetic lethal experiments. Elegant studies have recently shown that MYC, cyclin-dependent kinase 4 (CDK4) and CRAF are crucial KRAS effectors that can lead to synthetic lethality when genetically inactivated in tumours with activated KRAS[130–134].

The assessment of immunotherapeutics and the dynamic interactions between tumour cells and their microenvironment using GEMMs (which are immunocompetent) is another emerging research direction. Experiments of particular interest include gene expression and pathway activation profiles for each cell type within the tumour; genotype- or treatment-dependent influences on the tumour microenvironment; and effects of individual or combination therapies on tumour cells, immune cells and other cell types within the tumour microenvironment.

Patient-derived xenograft (PDX) models provide an alternative and complementary method to GEMMs to address human–murine differences and allow for expansion of patient material to perform assays such as metabolomic and serial transplantation[135]. A ‘humanized’ lung and even a ‘humanized’ immune system in the mouse might offer a more accurate means to model NSCLC.

Box 2

Lung stem and progenitor cell populations

Genetic lineage tracing and cell biology approaches have shown that the murine lung contains region-specific stem and progenitor cell populations that respond to local injury. Basal cells function as stem cells for the trachea, main bronchi and upper airways, where they can replace the `pseudostratified epithelium`, including secretory club cells (previously known as Clara cells), mucus-producing goblet cells and ciliated cells[136–138]. In more distal airways, club cells are a self-renewing population that maintains the ciliated cells[139], and subsets of club cells, such as bronchiolar progenitors, can give rise to ciliated and club cell lineages after injury[140,141]. In the alveolar space, where gas exchange is carried out by alveolar epithelial type 1 (AT1) cells, the surfactant-expressing AT2 cells can function as stem cells[60,142]. Another alveolar cell population, expressing α6β4 integrin, can also produce alveolar epithelia[142,143]. Bronchioalveolar stem cells (BASCs), which reside between the airway and alveolar space, can give rise to both epithelial lineages[56,114,144–146]. Murine proximal and distal lung stem cells can be isolated by fluorescence-activated cell sorting that uses different cell surface markers and can be grown in three-dimensional culture systems to study their differentiation potential[114,136,141,147]. Basal cells can be isolated from mouse or human lung on the basis of their expression of nerve growth factor receptor (NGFR)[136,148], and AT2 cells can be purified from distal lung — most recently with the marker HTII-280 (REF. [142]). Several other human lung stem cell populations have been reported in the human lung, but their roles have been controversial[149], and this points to the characterization of human lung stem and progenitor cells as an important area for future research. Furthermore, precisely how these cell types change their lineage potential in the face of oncogenic insult coupled with injury is unknown and is likely to influence tumorigenesis; injury and transformation might substantially alter plasticity[53]. A better understanding of lung stem and progenitor cells and methods for their analysis would open up new ways to explore the cellular origins of lung tumorigenesis.

Box 3

Patient stratification

Stratification and treatment selection for patients with non-small-cell lung cancer (NSCLC) heavily relies on radiographical and pathological evaluation in standard clinical practice. In recent years, molecular diagnostic platforms have been gradually introduced into this process. Today, many cancer centres and hospitals have adopted some degree of genetic diagnosis. Commonly accepted oncogenic driver mutations, including *KRAS*, epidermal growth factor receptor (*EGFR*), anaplastic lymphoma kinase (*ALK*), *ROS* and *BRAF*, are being sequenced and detected as a standard diagnosis procedure. Increasingly, mutation-based decision-making procedures are being integrated in the clinic, and we expect that additional novel technology platforms that stratify tumours according to the specific metabolome, epigenome and immune profile of each patient will be applied in the clinic in the near future. The anticipated challenge is how best to verify and use the vast amounts of sequencing information for translation to the clinic. Ongoing efforts seek to optimize data mining that will link existing genomic and biological data with clinical databases. In 2013, the Broad Institute, Cambridge, Massachusetts, USA, launched a global alliance that intends to share genomic and clinical data. A similar effort at Vanderbilt University, Nashville, Tennessee, USA, which is mediated by a publicly accessible website (My Cancer Genome), emphasizes the clinical application of cancer research. Worldwide efforts, such as the International Cancer Genome Consortium (ICGC) and the Catalogue of Somatic Mutations in Cancer (COSMIC) from the Sanger Institute, Hinxton, UK, and joint efforts in European countries to establish organoid cultures from primary tumours or biopsies from patients are also under way. Despite these independent efforts to integrate data sets, a more organized programme is needed on the national and international levels. The US National Center for Biotechnology Information recently initiated whole-genome sequencing to identify rare, druggable oncogenic events in patients who showed isolated but marked responses to certain drugs; this may represent the first exploratory step towards an integrated programme.

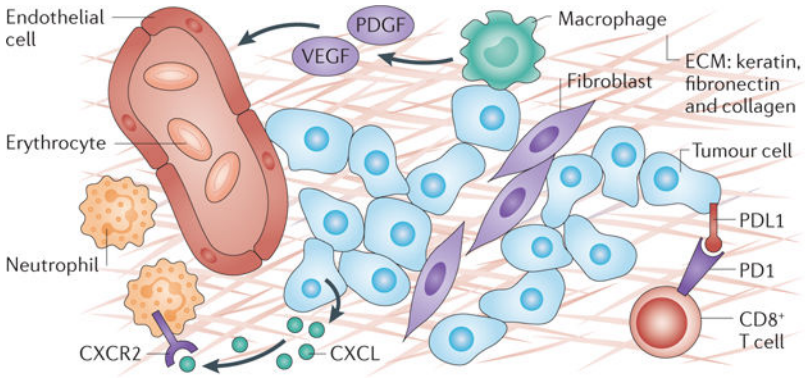

Figure 1. The lung cancer microenvironment

The tumour microenvironment, including endothelial cells, fibroblasts and myeloid cells, among others, has important roles in determining the characteristics of lung tumours. It is likely that a combination of the cell of origin, genetic alterations and microenvironmental factors all contribute to the lineage identity of lung tumours. Extracellular matrix (ECM), which often consists of keratins in lung squamous cell carcinoma and fibronectin in desmoplastic lung adenocarcinomas, gives structural support to tumour cells and is associated with tumour-associated fibroblasts. Blood vessels are newly formed at the tumour site by recruitment of endothelial cells via platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), among others. As the blood and lymphatic vessels form, numerous blood cells, including macrophages, neutrophils, T cells and B cells, home to the tumours. In particular, tumours can recruit neutrophils through secretion of CXC-chemokine ligand (CXCL) family members, which bind to the neutrophil receptor CXCR2. In addition, tumour cells often express immune checkpoint molecules, such as programmed cell death 1 ligand 1 (PDL1), to attenuate a cytotoxic response from T cells. PD1, programmed cell death 1.

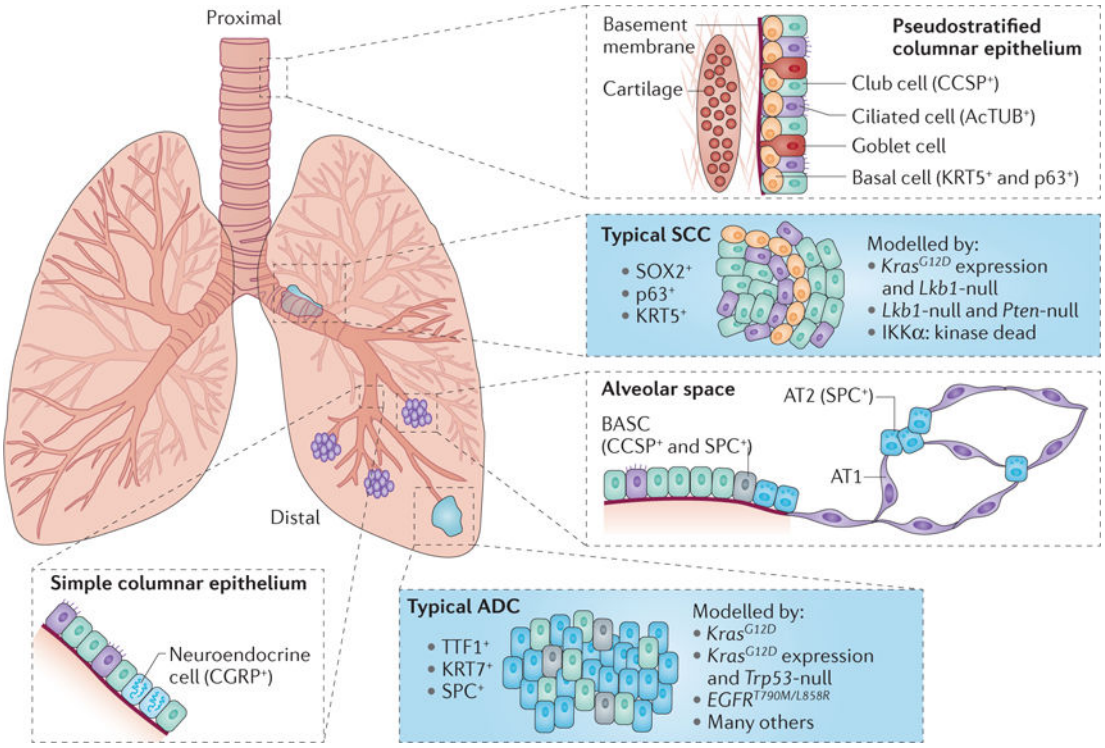

Figure 2. A diagram of proximal and distal lung cells, indicating markers that are retained in carcinomas and putative squamous cell carcinoma (SCC) and adenocarcinoma (ADC) cells of origin

Diverse lung stem or progenitor cell populations are thought to have the ability to drive lung oncogenesis in different contexts. In the proximal lung, the tracheal basal cell has been proposed to be the cell of origin for lung SCC. The evidence for this relationship includes the expression of p63, SRY-box 2 (SOX2) and keratin 5 (KRT5) within the basal cells, squamous metaplasia of the basal cells (common in smokers), and squamous cell carcinomas. Squamous tumours are modelled in mice by *Kras*^{G12D} expression and liver kinase B1 (*Lkb1*) knockout (20% of lesions are squamous), knocking in a germline dominant-negative kinase-dead inhibitor of nuclear factor-κB kinase subunit-α (IKKα) and knocking out both *Lkb1* and *Pten* (100% of lesions are squamous for the second two models). Two bronchiolar cell populations, the bronchiolar progenitor cells and the bronchioalveolar stem cells (BASCs) may also be able to give rise to tumours with squamous characteristics, although experimental lineage tracing is needed to confirm this theory. ADCs can be modelled by *Kras*^{G12D} expression (long latency), *Kras*^{G12D} expression and *Trp53*-null, and epidermal growth factor receptor (*EGFR*)$^{T790M/L858R}$, among other genetic models, and they are thought to arise from more proximal airway cells. These tumours often retain characteristics of proximal airways, such as the expression of surfactant protein C (SPC), KRT7 and thyroid transcription factor 1 (TTF1). Again, BASCs or bronchiolar progenitor cells, which are able to give rise to alveolar lineages after lung injury, may likewise be able to give rise to tumours with alveolar characteristics. AcTUB, acetylated tubulin; AT, alveolar epithelial type; CCSP, club cell secretory protein; CGRP, calcitonin gene-related peptide.

Table 1

Potential important alterations in ADC and SCC

Gene	Status (M, C or F)*	Frequency (%)		Available GEMMs	Currently available targeted therapies	Selected potential targeted therapies	Refs	
		ADC	SCC				Preclinical evidence	Clinical evidence
Receptor tyrosine kinases								
EGFR	M or C	10 (M)	2–3	L858R, Del19, T790M and Ins20	Erlotinib, gefitinib and afatinib	AZD9291, CO-1686 and HM61713	126	11
FGFR1	C	N/A	20	N/A	N/A	Dovitinib, ponatinib, AZD4547 and BGJ398	150	22
FGFR2	M or C	3 (M)	3	N/A	N/A	Dovitinib, ponatinib, AZD4547 and BGJ398	151	20
ALK	F	3–5	<1	ALK fusion, L1196M and F1174L	Crizotinib and ceritinib	AP26113, alectinib, ganetespib and PF-06463922	125	18
MET	C	2–4	N/A	Overexpression	Crizotinib	Tivantinib, cabozantinib, INC280 and onartuzumab	152	14
ROS1	F	1–2	N/A	N/A	Crizotinib	PF-06463922	153	17
NTRK1	F	1–2	N/A	N/A	N/A	Crizotinib and lestaurtinib	21	21
RET	F	1	N/A	N/A	N/A	Carbozantinib and vandetanib	154	16
HER2	M or C	2–4 (M)	N/A	HER2-YVMA insertion	N/A	Neratinib, afatinib, lapatinib and trastuzumab	155	19
DDR2	M	N/A	2–3	N/A	N/A	Dasatinib	27	27
PDGFRA	M	6–7	4	N/A	N/A	Sunitinib	156	28
Signalling								
KRAS	M	15–25	1–2	G12D, G12C and G12V	N/A	Selumetinib plus docetaxel combination	157	158
NF1	M	12	10	Null	N/A		159	28
BRAF	M	1–6	4–5	V600E	N/A	Vemurafenib, dabrafenib and trametinib	N/A	160
PIK3CA	M	5	15	p110α	N/A	BEZ235, BKM120 and GDC0941	99	161
MEK1	M	1	N/A	N/A	N/A	Selumetinib and trametinib	N/A	162
NOTCH1	M	8	1	Conditional null	N/A	N/A	163	164
Epigenetic factors								
MLL2	M	9	20	N/A	N/A	N/A	165	28

Gene	Status (M, C or F)*	Frequency (%)		Available GEMMs	Currently available targeted therapies	Selected potential targeted therapies	Refs	
		ADC	SCC				Preclinical evidence	Clinical evidence
EZH2	M	2	2	N/A	N/A	N/A	166	28
TET2	M	3	2	N/A	N/A	N/A	167	28
DNMT3A	M	4	1	N/A	N/A	N/A	168	28
Transcription factors								
SOX2	C	6	65	Overexpression	N/A	N/A	7	103
MYC	C	25	N/A	Overexpression	N/A	N/A	133	104
Proteolysis								
KEAP1	M	17	12	N/A	N/A	N/A	169	170
Cell cycle								
CDKN2A	M	7	15	Null	N/A	N/A	171	172
Ligand								
NRG1	F	<1	N/A	N/A	N/A	N/A	15	15
Tumour suppressor								
TP53	M	52	79	Conditional null and R172H	N/A	N/A	98	173
LKB1	M	9	2	Conditional null	N/A	N/A	65	174
PTEN	M	2	8	Conditional null	N/A	BEZ235, BKM120 and GDC0941	175	176

ADC, adenocarcinoma; *ALK*, anaplastic lymphoma kinase; *CDKN2A*, cyclin-dependent kinase inhibitor 2A (which encodes INK4A and ARF); *DDR2*, discoidin domain-containing receptor 2; Del19, *EGFR* exon 19 deletion; *DNMT3A*, DNA (cytosine-5-)-methyltransferase 3α; *EGFR*, epidermal growth factor receptor; *EZH2*, enhancer of zeste homologue 2; *FGFR1*, fibroblast growth factor receptor 1; GEMM, genetically engineered mouse model; Ins20, *EGFR* exon 20 insertion; *KEAP1*, kelch-like ECH-associated protein 1; *LKB1*, liver kinase B1; *MLL2*, mixed-lineage leukaemia 2; N/A, not available; *NF1*, neurofibromin 1; *NRG1*, neuregulin 1; *NTRK1*, neurotrophic tyrosine kinase, receptor, type 1; *PDGFRA*, platelet-derived growth factor receptor-α; *PIK3CA*, PI3K catalytic subunit-α; SCC, squamous cell carcinoma; *SOX2*, SRY-box 2; *TET2*, TET methylcytosine dioxygenase 2.

*Status refers to mechanisms by which each gene is altered in tumours — mutation (M), copy number gain (C) or fusion (F).

Nat Rev Cancer. Author manuscript; available in PMC 2017 December 04.