

Chromosomal and genomic changes in lung cancer

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Lung cancer is a complex spectrum of diseases characterized by extensive genomic instability, which can be detected among both histological subtypes and different foci within a tumor. Conventional and cutting edge investigative technologies have uncovered scores of genomic changes in individual specimens that have been used to characterize specific molecular subtypes. Oncogenes with predominant roles in lung cancer include *EGFR*, *MYC* and *RAS* family members, *PIK3CA*, *NKX2-1* and *ALK*; tumor suppressor genes include *TP53*, *RB1*, *CDKN2*, and a cluster of genes mapped at 3p. MicroRNA regulators also have been linked to lung cancer. The functional role of the recurrent genomic changes in lung tumors has been explored, which has led to a better understanding of cell growth, differentiation and apoptotic pathways. Additionally, this knowledge has supported the development of novel therapeutics and translational tools for selection of patients for personalized therapy.

Introduction

Lung cancer, comprised of two major clinico-pathological categories small-cell (SCLC) and non-small-cell lung carcinoma (NSCLC), is the leading cause of cancer-related morbidity and mortality worldwide.¹ SCLC accounts for less than 20% of lung tumors, displays neuroendocrine features and has a propensity for rapid growth and early metastasis. NSCLC represents the vast majority of these tumors and includes adenocarcinoma and squamous cell carcinoma, the two most common histological subtypes. Lung cancers are characterized by extensive genomic instability, which can be detected among both histological subtypes and among different foci within a tumor. The genomic changes occur at different levels, from mutations in single or few nucleotides to gains or losses of entire chromosomes. Some mutations are completely innocuous, but many of genomic events are responsible for dramatic functional changes and involve the core of lung carcinogenesis. In this article, we review relevant chromosomal and genomic alterations in lung cancer and discuss recent

findings that have contributed to an understanding of their molecular profiles and the development of strategies for earlier diagnosis and more efficient therapies.

Chromosomal Rearrangements in Lung Cancer: What is Known and How it Impacts Gene Expression

Usually lung carcinomas are highly aneusomic, with gains and losses of entire chromosomes or large chromosome regions. These tumors also exhibit simple and complex structural rearrangements responsible for alterations in transcription and protein expression. Included are variations in gene copy number due to deletions, duplications or amplifications, and gene fusions driven by insertions, inversions and translocations. Conventional cytogenetic methods, such as G-banding, were fundamental for initial discoveries on molecular mechanisms of lung carcinogenesis, but had limited utility in instances of cryptic or very complex rearrangements. The advent of molecular cytogenetic strategies in the early 1990s, such as multiplex FISH (M-FISH),² spectral karyotyping (SKY)³ and comparative genomic hybridization (CGH)⁴ have increased the accuracy of identifying chromosomal rearrangements (Fig. 1A and B), but these approaches were still limited by low resolution (5–10 megabases). New technological advances and the availability of genomic resources in the last decade have fostered the shift to microarray-based platforms, which has progressed from using only a few hundred DNA clones,^{5,6} to mining the entire genome for copy number variants at the 1 Mb resolution,⁷ and more recently selected analyses at the nucleotide level.^{8,9} Although high resolution platforms have been largely used to identify genomic rearrangements in lung cancer, intra-tumor heterogeneity still poses a challenge. Chromosomal abnormalities detected by these new technologies have been independently validated by other high-resolution laboratory approaches, such as fluorescence in situ hybridization (FISH) and polymerase chain reaction (PCR)-based techniques. Both are able to accurately define specific genomic regions involved in rearrangements and the PCR-based approach has high throughput. On the other hand, FISH has the critical advantage of investigating target phenomena in single cells “in situ” and of preserving the original tissue architecture. Ultimately, it is the combination of multiple technical approaches that provides the most powerful strategy for understanding the molecular pathways underlying the lung tumor development.

The first recurrent chromosomal abnormalities to be recognized in lung cancer were 3p deletions, identified by classical

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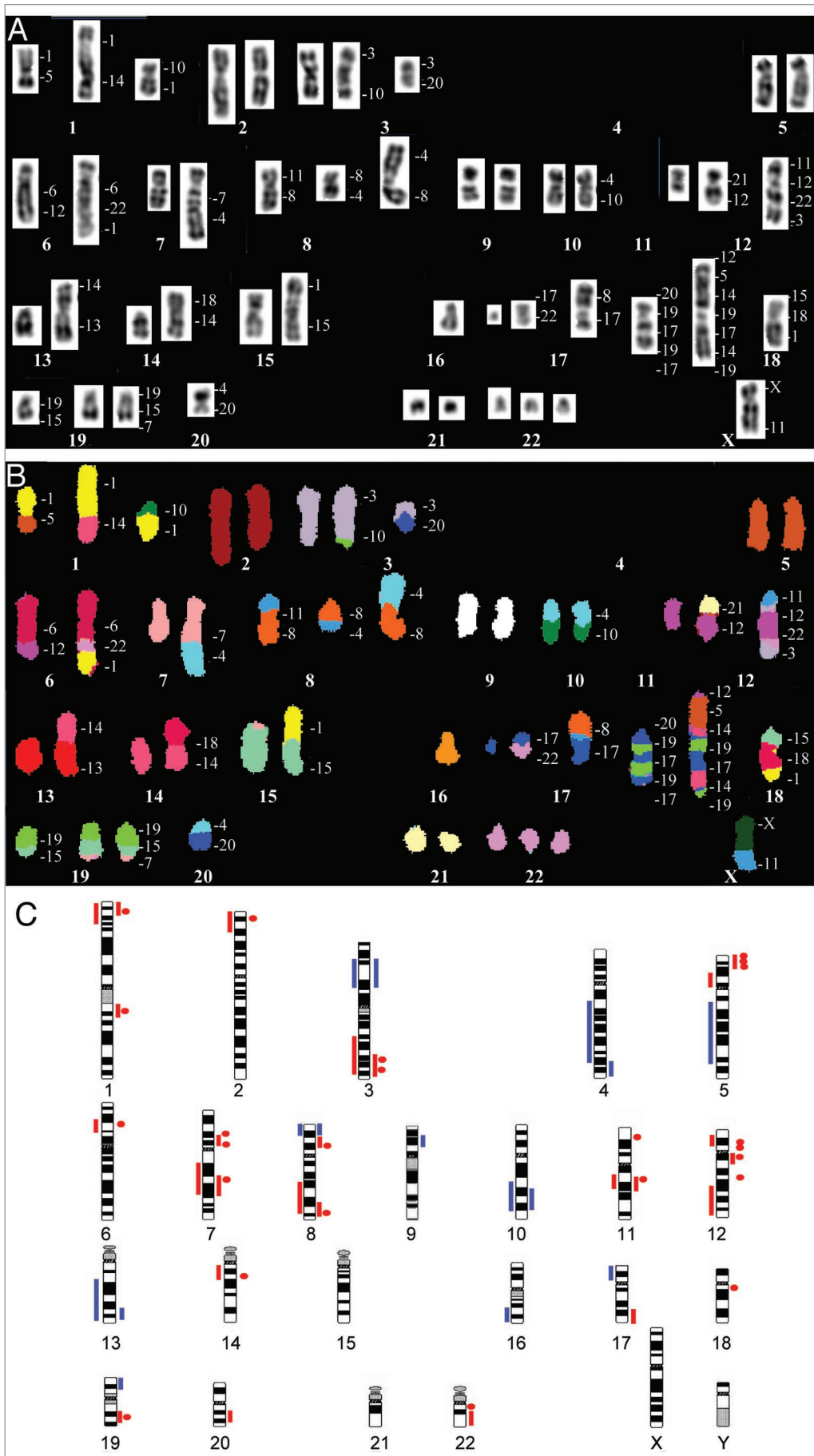


Figure 1. (A and B) Spectral karyotyping (SKY) of a lung adenocarcinoma showing numerous numerical and structural chromosome changes. The inverted-DAPI image is shown in (A) and the classified image with the pseudo-colors is shown in (B). The specimen was near-diploid, with rearrangements involving most chromosomes. In translocations, the origin of the material is listed on the right of the chromosomes. (C) Summary of genomic imbalances reported in lung cancer (reviewed in ref. 11). Small cell lung cancer (SCLC) is represented on the left of the chromosome ideograms, non-small cell lung cancer (NSCLC) is represented on the right. Copy number gain is represented by red bars, focal amplification by red dots and copy number loss by blue bars.

karyotyping in SCLC.¹⁰ For more than a decade, little new data were reported. It was only with the advent of M-FISH, SKY and CGH that cryptic rearrangements were detected, marker chromosomes were recognized and breakpoints were refined, providing a basis for the search of genes potentially deregulated and associated with tumor initiation and progression. A more detailed picture of genomic copy number variation in lung cancer was achieved recently with the array-based analyses and a summary of current data on gains and losses is presented in **Figure 1C** (reviewed in ref. 11). Analyses in more than 70 SCLC and 800 NSCLC cell lines and primary tumors identified important recurrent genomic changes, such as high-amplitude focal amplifications involving members of the MYC family (*MYCL1*, *MYCN* and *MYC*), participants in EGFR pathways (*EGFR*, *PIK3CA*, *KRAS*), and other genes controlling cell proliferation, such as *FGFR1*, *TP63*, *TERT*, *CCND1*, *CCNE1* and *NKX2-1*. These data have contributed to a growing body of evidence supporting the hypothesis that multiple cooperating oncogenes are involved in amplification events, apparently in non-random frequency. Importantly, several studies have shown that the expression of genes located in chromosomal regions involved in gains or losses varies consistently with the DNA copy number.^{12,13} Altogether, these findings have important implications for the design of functional genomic studies aimed at identifying cancer-relevant genes, since single-gene assays will not uncover activities that rely on interactions among multiple collaborating genes.

Growth Signaling and Apoptotic Pathways: The Balance of Stimulatory and Inhibitory Genes

In clinically evident lung cancer, genomic changes involve both tumor suppressor genes and oncogenes. Tumor suppressor genes are commonly inactivated by a combination of genetic mechanisms such as point mutations, chromosomal rearrangements and mitotic recombinations, and by epigenetic events like methylation of promoter regions.¹⁴ The major tumor suppressor genes involved in lung cancer are *TP53* (17p13.1), *RBI* (13q14.11), *CDKN2* (*p16^{INK4a}* or *MST1*, 9p21), and several genes located at 3p. *TP53* is well known for its key role in the negative regulation of the cell cycle G₁/S phase transition and for being a gatekeeper for apoptosis.^{14,15} Mutations and overexpression of *TP53* are almost universal in lung cancer and associated with smoking and more aggressive tumors.¹⁶⁻¹⁸ *RBI* controls the G₁/S transition through E2F^{19,20} and may also be inactivated by nonsense mutations or splicing abnormalities, most commonly in SCLC. *CDKN2/p16/MTS1* encodes a CDK4 inhibitor and is frequently abnormal in NSCLC (16% to 100%).²¹ *CDKN2* hypermethylation predicts a poor 5-year survival rate in resectable NSCLC²² and early recurrence in resected stage I NSCLC.²³ Partial deletion of 3p occurs in almost all analyzed SCLCs and NSCLCs²⁴ and encompasses numerous genes identified as tumor suppressors including *FHIT* (3p14.2), *RASSF1* (3p21.3), *TUSC2* (*FUS1*, 3p21.3), *SEMA3B* (3p21.3), *SEMA3F* (3p21.3) and *MLH1* (3p22.3). Allelic imbalance of *FHIT* is associated with chromosomal deletions^{25,26} while *RASSF1* and the mismatch repair gene *MLH1* are inactivated by promoter hypermethylation.²⁷⁻²⁹ *TUSC2*,³⁰ *SEMA3F* and

SEMA3B transcripts³¹ are recurrently underrepresented in lung cancers and the *SEMA3s* were found to be targets of *TP53*,³² which suggests they could be activated during DNA damage or other stress responses.

Numerous proto-oncogenes contribute to lung cancer pathogenesis when constitutively activated, such as the members of the *EGFR* (ERBB), *MYC* and *RAS* families, as well as *PIK3CA*, *NKX2-1* and *ALK*. The activation of proto-oncogenes frequently occurs by genetic mutations (*KRAS*, *EGFR*, and *PIK3CA*), amplifications (*MYC*, *EGFR*, *HER2*, *PIK3CA*, *NKX2-1*), and chromosomal rearrangements, such as translocations and inversions that place these genes under the regulation of constitutively activated genes (*MYC*) or create chimeric proteins (*ALK-EML4*).

Among the most important factors for lung tumor growth and proliferation are the tyrosine kinase receptors of the ERBB family, which are coded by the genes epidermal growth factor receptor (*EGFR*, 7p12), *ERBB2* (HER2/neu, 17q12), *ERBB3* (12q13) and *ERBB4* (2q33.3). The EGFR protein is overexpressed in the majority of lung carcinomas.^{32,33} Activating mutations in the *EGFR* tyrosine kinase domain prevail in lung cancer patients of East Asian ethnicity, never-smokers, females, and NSCLC with adenocarcinoma histology.³⁴⁻³⁷ The *EGFR* gene is amplified in approximately 10% to 15% of advanced NSCLC.^{34,38-42} Phosphorylation of *EGFR* activates signaling to cell proliferation and survival via RAS/MAPK and PIK3CA/AKT pathways.⁴³ Both EGFR protein overexpression and gene amplification have shown a trend towards poor prognosis^{33,42} while activating mutations have been associated with better prognosis and indolent disease.^{44,45} The other members of the EGFR family are also important, although less critical. Overexpression of *ERBB2* ranges from 10 to 30% in NSCLC;⁴⁶ *ERBB2* gene amplification is less common (6 to 20%)^{47,48} and activating mutations are rare.⁴⁷ These features are associated with poor survival and resistance to EGFR tyrosine kinase inhibitors (TKIs) in cases with clinical and biological features of sensitivity to such treatment.⁴⁷ *ERBB3* is overexpressed in 20 to 60% of lung tumors, especially squamous cell carcinomas,⁴⁹ is genomically amplified in 5% without histology subtype specification,⁵⁰ and is also correlated with shorter survival.⁵¹ *ERBB4* is still poorly understood and seems to infrequently (<3%) harbor mutations in NSCLC.⁵²

The genes of the *RAS* family (*HRAS* at 11p15.1, *KRAS* at 12p12.1, and *NRAS* at 1p13.2) encode for highly homologous G-proteins located at the inner surface of the cell membrane with essential roles in signal transduction pathways involved in differentiation, proliferation and survival. In lung cancer, *KRAS* is more frequently mutated than *HRAS* and *NRAS*.⁵³ The mutant proteins permanently fixed in the active position and constitutively activate downstream signaling pathways, including *BRAF*, *MAPK* and *PI3K/AKT*.⁵⁴ *KRAS* mutations prevail in large-cell carcinomas and adenocarcinomas (20–30%). *KRAS* and *EGFR* mutations are almost completely mutually exclusive.³⁷ *KRAS* mutation has been reported as a negative prognostic factor for survival in NSCLC⁵⁵ and for not responding to EGFR tyrosine kinase inhibitors.⁵⁶ Other downstream effectors of the RAS pathway, such as *BRAF*, which encodes a serine-threonine kinase activated by point mutation, are infrequently mutated in lung cancer (<5%) and are

likely to have a lesser relevant role in the pathogenesis of these carcinomas.⁵⁷

The *MYC* family of genes (*MYC* at 8q24.1, *MYCN* at 2p24 and *MYCL1* at 1p34) encodes basic-helix-loop-helix zipper (bHLH) transcription factors that, after dimerization with MYC-associated factor X (Max), binds to E-box motifs (CACGTG, CANNTG) and stimulates the transcription of various target genes relevant for cell growth, differentiation and apoptosis.⁵⁸ Additionally, there is increasing evidence that the *MYC* genes bind ubiquitously throughout the genome, apparently to genomic sites at up to 15% of all cellular genes, which hints at a potential non-transcriptional function.⁵⁹ The alternative model for the role of *MYC* in cell growth and tumorigenesis is corroborated by studies showing that *MYC* promotes DNA replication via non-transcriptional mechanisms and its deregulation causes DNA damage predominantly during the S-phase.⁶⁰ *MYC* was shown to be the most frequently amplified oncogene in lung cancer cell lines (28% of 53 investigated lines).⁶¹ Amplification and overexpression of *MYC* genes occurs in more than 20% of SCLCs and NSCLCs in association with resistance to chemotherapy, tumor progression and worse prognosis.⁶²

The PI3K-PTEEN-AKT signaling pathway transmits a strong cell survival signal through interactions between cell surface receptors (IGF1R, PDGF, EGFR), extracellular ligands (EGF, TGF α), and the recruitment of class I PI3Ks and specific intracellular proteins (PDK-1, Akt/PKB) by mechanisms regulated by *PTEEN* and *AKT*.⁶³ The p110 α catalytic subunit of PI3Ks is coded by the *PI3KCA* gene (3q26) and there is increasing evidence that constitutive activation of the PI3K pathways in lung cancer occurs as a consequence of *PIK3CA* mutation or amplification. *PIK3CA* genomic gain detected by FISH was reported in 43% of lung cancers with a preference for squamous cell carcinoma⁶⁴ and overexpression of phosphorylated Akt has been observed in approximately 50% of advanced NSCLC.³⁴

It has been postulated that genetic alterations that directly interfere with transcriptional networks regulating lung development may be a more common feature of lung cancer than previously realized.⁶⁵ Supporting this was the recent finding of amplification of the homeobox transcription factor, *NKX2-1* (14q13.3),^{65,66} which plays a master role in induction and maintenance of lung and thyroid morphogenesis and differentiation of epithelial cell lineages.⁶⁷ Gain at 14q13.3 was present in more than 10% of lung cancer specimens and was significantly more frequent in adenocarcinomas.⁶⁸

An interesting example of activation of a tyrosine kinase by gene fusion due to structural chromosomal rearrangements involves *ALK*. The *EML4-ALK* fusion (Fig. 2A) resulting from inversion in chromosome 2p and the *TFG-ALK* and *KIF5B-ALK* fusions resulting from the translocations t(2;3)(p23;q21) and t(2;10)(p23.2;p11.22), respectively, occur in approximately 4% of NSCLC and comprise a newly defined molecular subtype.⁶⁹⁻⁷¹ In these gene rearrangements, the promoter of the 5' partner gene controls transcription of the resulting fusion gene. The fusion partner typically contains an oligomerization domain that mediates constitutive dimerization, subsequent autophosphorylation and activation of the *ALK* kinase in the absence of ligand, which

is important for both tumorigenesis and tumor maintenance. These *ALK* gene rearrangements appear to be more common in lung adenocarcinomas from never or light smokers whose tumors are wild-type for *EGFR* and *KRAS*.^{72,73}

Genomic Changes and Target Therapy to NSCLC

The genomic changes in proto-oncogenes are important drivers for therapeutic strategies. Observations that inactivation of a few or even a single oncogene was sufficient to induce a sustained tumor regression have supported the “oncogene-addiction” hypothesis. The model proposes that tumors may become irrevocably addicted to the oncogene that initiated tumorigenesis and a sudden interruption of its activity shifts the balance towards proliferative arrest and apoptosis.⁷⁴ The *ALK*-driven cancers, for example, have shown excellent response to specific *ALK* inhibitors in Phase I clinical trials.⁷² The status of the *EGFR* gene has also proved to be a powerful predictive marker for targeted therapy. Not surprisingly, patients with *EGFR* activating mutations and high copy numbers are more sensitive to *EGFR* tyrosine kinase inhibitors, such as gefitinib and erlotinib.^{34-36,40,43,75} NSCLC patients with *EGFR* gene amplification or high-level genomic gain by chromosomal aneusomy (Fig. 2B and C) have also shown higher sensitivity to the monoclonal anti-*EGFR* antibody, cetuximab.³⁹ Amplification of other genes, such as *MYC* and *EIF3H*, has also been associated with better response to *EGFR* TKIs⁷⁶ although the mechanisms involved are still to be determined. Another interesting example implicates *MET* (7q31.2). Enhanced *MET* regulation leads to oncogenic changes including cell proliferation, reduced apoptosis, angiogenesis, altered cytoskeletal function and metastasis. Mutations in the tyrosine kinase domain and *MET* gene amplification are uncommon in unselected NSCLC^{77,78} but *MET* gene amplification (Fig. 2D) was found to be a major mechanism by which lung tumors overcome *EGFR* inhibition and develop resistance to *EGFR* TKIs.^{77,79}

MicroRNAs as Novel Regulators in Lung Cancer

Interesting new players in carcinogenesis are the microRNAs (miRNAs), a recently identified class of highly conserved, endogenous, non-coding RNAs that regulate gene expression in a sequence-specific manner.^{80,81} In their mature form, miRNAs are 19 to 25 nucleotides in length and are predicted to regulate as many as 300 to 400 messenger RNA (mRNA) targets. These molecules are of particular importance in cancer biology because many have been shown to be altered by amplification or deletion, a hallmark of the cancer genome.⁸² Furthermore, miRNAs are better classifiers of tissue origin for cancer cell lines or tumor tissues than are mRNA biomarkers⁸³ and signatures of miRNA expression can define molecular subsets of tumors^{84,85} and predict outcome.^{82,86,87} The tissue specificity of miRNA expression, their incredible stability and their ability to regulate multiple mRNA targets make them attractive as a novel class of biomarkers in lung cancer.

There are numerous studies focusing on miRNAs and lung cancer with relevant results. Overexpressed microRNAs are

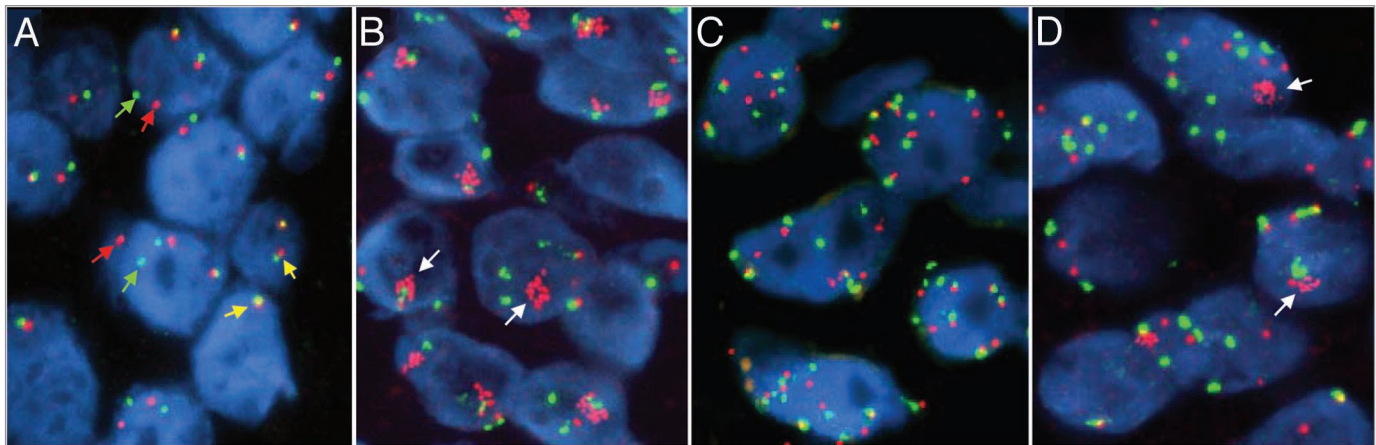


Figure 2. Fluorescence in situ hybridization (FISH) images of sections of non-small cell lung cancer hybridized with the *ALK* Break-Apart (A), the *EGFR*/CEP 7 (B and C) and the *MET*/CEP 7 (C) probe sets. *ALK* Break-Apart and the *EGFR*/CEP 7 probe are commercially available (Abbott Molecular), *MET*/CEP 7 is a "homebrew" probe. In each panel, the chromatin from the nuclei is stained with DAPI (blue color). In (A), the *ALK* Break Apart FISH probe includes DNA sequences contiguous to the 3' end of *ALK* labeled in red and sequences of the 5' end of *ALK* labeled in green. (A) Shows an adenocarcinoma specimen harboring the *EML4-ALK* fusion that is detected as split red and green signals (red and green arrows). The fused red/green signals (yellow arrows) indicate native status of the *ALK* gene. In (B and C), DNA sequences encompassing the *EGFR* gene are labeled in red and the centromere 7 control is labeled in green. In normal copies of chromosome 7, these two signals are physically close since *EGFR* maps at 7p12. (B) Shows a lung adenocarcinoma specimen harboring amplification of the *EGFR* gene (clusters of red spots indicated by the white arrows). (C) Shows a squamous cell carcinoma exhibiting copy number gain for both the *EGFR* gene and the control CEP 7. In (D), sequences encompassing the *MET* gene were labeled in red and the centromere 7 control is in green. (D) Shows an adenocarcinoma specimen exhibiting amplification of the *MET* gene (clusters of red spots indicated by the white arrows).

expected to function as oncogenes and one such example involves the *hsa-mir-17-92* cluster. This cluster comprises more than forty distinct miRNAs residing in an intron of *MIRHG1* at 13q31.3, a gene that is markedly overexpressed and occasionally amplified in lung cancer.⁸⁸ The predicted targets for this miRNA cluster comprise a large number of genes, including the tumor suppressors *PTEN* and *RB2*.⁸⁹ Under the same principle, some mi-RNAs function as tumor suppressor genes. Downregulation of miRNA *hsa-let-7g* in 3p21.2 and miRNA *hsa-mir-128b* in 3p22 was recently found to be associated with overexpression of RAS and EGFR, respectively.⁹⁰ These findings provide a functional link between the first recurrent abnormality detected in lung cancer, deletion of 3p sequences, and deregulation of *KRAS* and *EGFR*. *Hsa-mir-128b* was demonstrated to directly regulate *EGFR* and, most importantly, its loss had a favorable impact in the sensitivity to *EGFR* TKIs, which was comparable to EGFR copy number gain, associated with significantly better disease control and longer survival. Amplification of oncogenes also regulates signaling pathways through miRNAs. For instance, *MYC* activates expression of the miRNA cluster on chromosome 13, and two of these miRNAs (*hsa-mir-17-5p* and *hsa-mir-20A*) negatively regulate

E2F.⁹¹ This association reveals the tightly controlled mechanism for activation of transcription and limitation of translation exert by *MYC* on *E2F*.

Conclusions

Lung cancer is a spectrum of diseases with numerous alterations in expression patterns resulting from acquired genetic and epigenetic mechanisms. Conventional and cutting-edge investigative technologies have detected scores of genomic changes in individual specimens. However, few of those changes are recurrent among large numbers of tumors, a characteristic that poses a challenge for the precise definition of molecular subtypes.

Studies focusing on the functional role of genomic changes in lung cancer are in dramatic expansion. Ultimately, these genomic discoveries are expected to contribute to a better understanding of cell growth, differentiation and death pathways, and to the development of novel therapeutics and translational tools for assessment of risk, early diagnosis and selection of patients for personalized therapy.

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