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The role of DNA methylation in the development and progression of lung adenocarcinoma

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Abstract. Lung cancer, caused by smoking in ∼87% of cases, is the leading cause of cancer death in the United States and Western Europe. Adenocarcinoma is now the most common type of lung cancer in men and women in the United States, and the histological subtype most frequently seen in never-smokers and former smokers. The increasing frequency of adenocarcinoma, which occurs more peripherally in the lung, is thought to be at least partially related to modifications in cigarette manufacturing that have led to a change in the depth of smoke inhalation. The rising incidence of lung adenocarcinoma and its lethal nature underline the importance of understanding the development and progression of this disease. Alterations in DNA methylation are recognized as key epigenetic changes in cancer, contributing to chromosomal instability through global hypomethylation, and aberrant gene expression through alterations in the methylation levels at promoter CpG islands. The identification of sequential changes in DNA methylation during progression and metastasis of lung adenocarcinoma, and the elucidation of their interplay with genetic changes, will broaden our molecular understanding of this disease, providing insights that may be applicable to the development of targeted drugs, as well as powerful markers for early detection and patient classification.

Keywords: AAH, adenocarcinoma, BAC, CpG island, DNA methylation, hypermethylation, hypomethylation

1. Introduction

Lung cancer is the leading cause of cancer death in the United States and Western Europe for both men and women, taking over 163,000 American lives in 2005 [1, 2]. Approximately 87% of lung cancer cases are caused by exposure to tobacco products [3]. Unfortunately, the marked decrease in smoking in the United States, observed for men in the 1970s and 1980s, appears to be leveling off [4], and in 2002, approximately 25% of men and over 20% of women in the United States smoked [4]. A roughly similar situation prevails in many West European countries. In addition, a large number of previous longtime smokers remain at high risk for lung cancer development for years after smoking cessation; a recent study of a prospective cohort of Iowa women showed that for adenocarcinoma, former smokers had an elevated risk for *up to 30 years* after quitting smoking [5]. Lung cancer is clinically subdivided into small cell lung cancer (SCLC; ∼15–20% of lung cancers), the most aggressive form of lung cancer, and non-small cell lung cancer (NSCLC), consisting of adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and miscellaneous other types such as carcinoids, pleomorphic and mixed carcinomas and a range of neuroendocrine cancers [6]. In the United States, adenocarcinoma has now surpassed squamous

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cell cancer as the most common histological subtype of lung cancer, and continues to increase worldwide, particularly in women [7–9]. Adenocarcinoma generally arises more peripherally in the lung. Its increased incidence is thought to be related at least in part to changes in the structure and composition of cigarettes (lower tar, lower nicotine, leading to deeper inhalation) [10, 11]. Adenocarcinoma is also the most common type of lung cancer in previous and never-smokers, and among young patients with adenocarcinoma, the percentage of women and non-smokers is unusually high [12]. Recently, certain human papilloma virus subtypes have been implicated in adenocarcinoma in non-smoking women in Asian countries (reviewed in [13]). These observations point to the importance of gaining a better understanding in the etiology of lung adenocarcinoma, and in the molecular changes underlying this disease.

The study of genetic mutations/deletions has provided important mechanistic insight into tumorigenesis in many different organs. However, it has recently become clear that *epi*genetic alterations also play an important role in cancer development. Epigenetic changes are heritable through cell division, and result in altered gene activity without any accompanying changes in the DNA sequence [14,15]. The most easily quantifiable and best characterized epigenetic alteration in cancer is a change in the pattern of DNA methylation. This review will be limited to the discussion of DNA methylation-based epigenetic alterations.

In mammals, DNA methylation predominantly consists of the addition of a methyl group to the 5 position of cytosine, primarily at CpG dinucleotides. This is a normal and essential enzymatic modification that is required for proper development of the organism (recently reviewed in [16]). DNA methylation is required for chromosomal stability, and is a powerful mechanism for maintaining the suppression of gene activity in vertebrates, for example in X-chromosome inactivation [14,17–20]. In cancer, the pattern of DNA methylation normally seen in healthy cells is altered; there is a *global* loss of methylation, associated with chromosomal instability, and *local* changes in methylation in clusters of CpGs, or CpG islands [16,20,21]. CpG islands are G:C- and CpG-rich areas of about 1 kb, often found near the gene promoter in the case of widely expressed genes [22–24]. During carcinogenesis, certain genes can become activated through loss of CpG island methylation, sometimes associated with loss of maternal or paternal imprinting [25]. However, a much more commonly observed alteration at CpG islands is *hyper*methylation, which is seen as a key mechanism for the inactivation of genes that, in a variety of ways, normally contribute to negative regulation of cell growth. The relationship between promoter methylation of cytosine residues and the transcriptional silencing of genes has been widely documented [18,19,26].

De novo methylation of 5' CpG islands is occasionally seen in normal somatic tissues, particularly in the case of certain genes that show a gradual increase in methylation during aging [27]. However, this is much more common in cancer: hypermethylation of CpG islands has been observed in virtually every type of human cancer that has been investigated [20,28, 29], including lung cancer (recently reviewed in [30–32]). Because $\sim$40% of human genes carry promoter CpG islands [33], CpG island hypo- and hypermethylation provides an enormous potential for epigenetic alterations in cancer, giving rise to phenotypic changes which, combined with genetic alterations, underlie cancer development and progression [25,28,29, 34,35]. Studies of DNA methylation in lung cancer to date strongly suggest that the analysis of DNA methylation profiles will be of great utility both for understanding the molecular basis of lung cancer development [36–39], and for developing tools for accurate and early lung cancer diagnosis and prognostication [21,30, 31]. In addition, the reversibility of DNA methylation suggests that clinical strategies might be developed to reactivate genes silenced by methylation [40].

Much current work still focuses on the important and gargantuan task of cataloging cancer-specific DNA methylation alterations in lung cancer, a task that is far from complete. However, recently some of the focus has shifted to the mapping of sequential DNA methylation changes during cancer initiation and progression, and the clarification of how epigenetic and genetic changes collude to promote cancer development. In colon adenocarcinoma, studies of cancer at different stages has allowed elegant sequential models of cancer progression to be developed [41,42]. In lung cancer such work is more challenging, as samples of early lesions are much more difficult to obtain. However, work from animal models combined with studies of human tissues, blood and sputum samples, have begun to yield insights into early and late DNA methylation changes [31]. Here we will discuss what is known about the timing of DNA methylation alterations in lung adenocarcinoma, and how these changes might interface with known genetic alterations. Although there are numerous reports of methylation in lung cancer in general, many lack information about histology, the relationship between methylation and tumor stage, or whether

methylation is present in non-tumor tissue. Since reports lacking any such information do not shed light on progressive methylation in developing lung adenocarcinoma, they will not be cited here. For a broader overview of DNA methylation in lung cancer the reader is referred to previously published reviews [30–32].

2. Development and progression of lung adenocarcinoma

In order to begin mapping out the chronology of epigenetic changes in the development and progression of lung adenocarcinoma, it is important to obtain an overview of the different stages leading to advanced lung adenocarcinoma. This knowledge, summarized in Fig. 1, will form the framework into which data about epigenetic alterations will be incorporated. The first step (Fig. 1, step 1) represents the acquisition of genetic or epigenetic changes that affect large sections of the lung. Environmental exposures are assumed to be to blame for these large regional (epi)genetic changes, which are referred to as field effects or defects [43–45] and which also have been called field "cancerization" [46]. Longtime smoking, which is considered to be the cause of lung cancer in ∼85% of cases, and other exposures such as asbestos and radiation, likely play a role in generating (epi)genetically altered fields [47]. More recently, certain strains of human papilloma virus have been implicated in lung cancer, in particular in non-smoking Asian women (reviewed in [13]), and these could likewise lead to (epi)genetic alterations. The influence of the environment on the epigenome is well illustrated by the demonstration that epigenetic differences arise in monozygotic twins during their lifetime [48]. Indeed, analysis of DNA methylation of histologically normal bronchial margins of lung cancer patients shows frequent hypermethylation similar to that seen in the tumors [43]. This is reminiscent of field defects showing DNA hypermethylation in colon cancer [49]. The fact that field defects are found in healthy-looking microdissected lung [50] argues against an inflammatory explanation, and its association with smoking reinforces the idea that the environment plays a strong role (see below).

Field defects would predispose cell populations in the lung to the development of microscopically visible precancerous lesions. This is precisely what is seen in ∼20% of lung cancer patients: they carry atypical adenomatous hyperplasia (AAH, Fig. 1, step 2) in their adjacent non-tumor lung tissue, sometimes as a multitude of individual tiny lesions [51]. AAH consists of runs of cuboidal cells lining slightly thickened alveolar walls (see Fig. 1 [52]). The suggestion that AAH is a precursor of lung adenocarcinoma is based on several observations. First, AAH occurs predominantly in lung adenocarcinoma patients; 72–81% of AAH lesions are found in patients with a primary adenocarcinoma [51,53,54]. Second, activating KRAS point mutations, which are found in ∼30% of lung adenocarcinomas [55], have been found with similar frequency in AAH in at least one study [56]. Third, analysis of X-chromosome inactivation in individual AAH lesions has shown that they are monoclonal, while similar sized reactive metaplastic lesions are not, indicating that AAH represents outgrowth of a single altered cell [57]. In addition, AAH lesions and contiguous bronchioalveolar carcinoma were also shown to be monoclonal [57]. It has been proposed that cancer stem cells could play a role in the development of early precancerous lesions in defective fields [25]. The recent identification of potential lung cancer stem cells that appear to be precursors for lung adenocarcinoma in a mouse model system lends credence to this idea [58,59].

AAH lesions vary in their cellularity and atypia, ranging from relatively low grade lesions to those with higher grade changes. The latter is presumably a progression of the former, and is very similar to localized non-mucinous bronchioalveolar carcinoma [51] (BAC, Fig. 1, step 3). According to the new WHO classification, BAC is defined as a non-invasive lesion [60]. Pure localized BACs are rare, as any invasive component must be completely excluded [61]. BAC is considered the likely intermediary stage between AAH and invasive adenocarcinoma [51,53,57,61,62].

Once invasion has developed within BAC, the lesion is considered adenocarcinoma (Fig. 1, step 4), and its progression is reflected in the clinically or pathologically assigned stage (Fig. 1, step 5). Staging is of key importance for the clinical management of patients, and to provide prognostic indicators [63]. Staging can be clinical (based on physical examination and imaging) or surgical-pathologic (based on surgery with accompanying lymph node dissection and pathologic description of the extent of tumor spread). The assigned stage is based on the TNM system: T is determined by the size of the tumor and/or its location (T1-3). N is determined by the involvement (or not) of lymph nodes (N0, no positive nodes, N1, involvement of peribronchial and/or ipsilateral hilar region nodes; N2, involvement of ipsilateral mediastinal or subcarinal nodes; N3, involvement of contralateral mediasti-

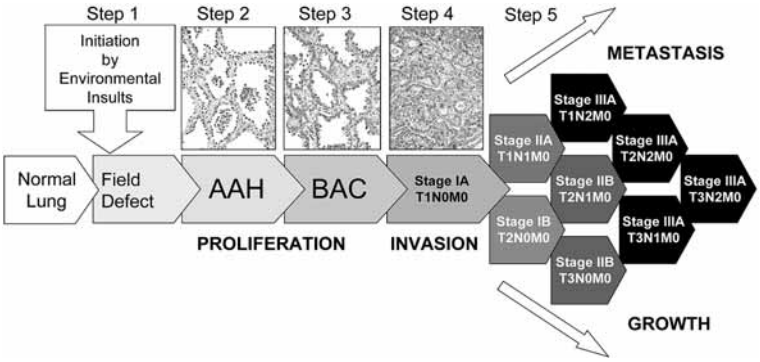

Fig. 1. Development and progression of lung adenocarcinoma. Step 1: Environmental exposures, such as tobacco smoke, radiation, asbestos, viral infection, etc. lead to histologically invisible changes that result in molecularly altered fields of cells (a process often referred to as "field cancerization"). Presumably, the response to environmental insults is modulated by the subject's genetic background. Environmental exposures can continue throughout all following steps, and are thought to stimulate progression. Step 2: Within these altered fields, additional (epi)genetic changes are acquired, leading to phenotypic changes in affected cells and the formation of small histologically visible lesions (AAH). Step 3: AAH lesions progress, acquiring further (epi)genetic changes, retaining alveolar structure, developing more cytological atypia but not yet becoming invasive, resulting in bronchioalveolar carcinoma. Step 4: (Epi)genetic changes continue, allowing cells within BAC to become invasive. Step 5: Further molecular alterations allow growth in size of adenocarcinoma and metastasis to nodes and eventually other organs.

nal or hilar nodes, or ipsilateral/contralateral scalene or supraclavicular nodes) [64]. M status is determined by nodal spread beyond N3 node stations and/or to distant organs (M0 none, M1 metastases).

The importance of studying the molecular changes in these different stages of lung adenocarcinoma is supported by two observations. The first is that lung cancer patient survival differs for the distinct stages [63]. Survival of each *pathologic* stage is better than its corresponding *clinical* stage, because clinically staged cases showing previously undetected lymph node metastases (N0) are moved up in stage upon discovery of involved nodes ("stage migration") [64]. Nevertheless, statistically significant differences in 5-year survival are seen between all early clinical stages except between IIA (T1N1M0) and IIB (T2N1M0), and *all* early pathologic stages [63]. In addition, statistically significant differences are seen between survival of all different N designations (clinical or pathologic) [64]. For example, for clinical N0 vs. N1, the 5-year survival is $\sim$40 vs. $\sim$20%, whereas for pathologic N0 vs. N1 it is $\sim$60 vs. $\sim$40%. A second reason why knowledge of molecular changes in the various stages is important, is that even in pathologic stage N0 disease, only $\sim$60% of patients will survive $\geqslant$5 years. Pathologic N0 patients, cumulatively referred to as stage I, fall into two sub-stages: IA, which is T1N0M0, and IB, which is T2N0M0. Stages IA and IB show statistically significant differences in 5-yr survival (67 and 57% respectively), but the basis for this difference remains unknown. It is assumed that molecular alterations, in other words, genetic and/or epigenetic changes, are responsible for these survival disparities. Patients who have successful resection of pathologic Stage I disease yet later succumb to the same disease are presumed to have had undetectable micrometastases at the time of surgery.

3. The interplay of genetic and epigenetic alterations

Genetic and epigenetic events both contribute to the development and progression of lung adenocarcinoma, and these changes have the potential to affect each other [25,65]. Epigenetic silencing of DNA repair genes such as MGMT or MLH1 might predispose cells to the acquisition of further genetic hits [49,66,67], while mutations in genes involved directly or indirectly in *de novo* or maintenance methylation could affect DNA methylation levels [68–72]. Dissecting the respective epigenetic and genetic contributions, and determining the timing with which (epi)genetic events occur during carcinogenesis, is one of the most exciting challenges in the field.

Our ability to construct timelines of (epi)genetic hits is hampered by two major obstacles. The first is that knowledge about the timing of these hits remains limited, because it is often unclear when these changes first appear. Indeed, the same alteration can occur at different times in different patients. We shall consider them "early" if they occur with a measurable frequency in precancerous tissue, even if they become more frequent at later stages. The second is that most (epi)genetic alterations will not be fully penetrant; in other words,

only a fraction of lesions or tumors will carry a particular molecular change. This is because *pathways* are the targets of (epi)genetic hits rather than individual genes [73]. The molecular pathways for cell survival, growth in response to signaling, the ability to proliferate in an anchorage independent fashion, etc. are complex and each involve a large number of factors. Distinct genes in a single pathway may be affected in different patients, giving rise to the same phenotypic result. Adding further complexity, (epi)genetic hits might be acquired at later stages in already disrupted pathways, perhaps even in the same gene, leading to a more profound deregulation. Thus, it is crucially important to see individual (epi)genetic events in a larger context, each as an alteration in a particular subsection of the cellular circuitry. We will take this into consideration as we begin to piece together the (epi)genetic puzzle of lung adenocarcinoma development and progression. Although many pieces of this puzzle are still missing, it will nevertheless provide a framework within which newly identified hits can be placed.

4. Early (epi)genetic changes: Histologically normal lung

As outlined in Fig. 1, the earliest pathways altered in cancer development would allow proliferation of certain cell groups and would provide fertile ground for subsequently arising lesions. These pathways would likely be related to growth control, differentiation and/or apoptosis. Because the earliest changes may not be visible at the histological level, they may be very difficult to characterize. One way to do so would be to ask: What kind of (epi)genetic changes are observed in the lung from areas adjacent to lung cancers, and how do these areas compare to regions further from the tumor? An autopsy study on the lungs of a cancer-free smoker showed an identical p53 mutation in 7 out of 10 widely separated sampled sites, but no such mutation in blood or non-pulmonary organs [74]. While this is an isolated case, and p53 mutations do not appear to be very common in lung adenocarcinoma, it suggests that somatic mutations can occur early and might spread widely throughout the lungs, perhaps through the migration of cancer stem cells.

Distinct regions of loss of heterozygosity (LOH) of chromosome 3p have been found in normal lung from many lung cancer patients [75,76], and microsatellite analysis has indicated multiple potentially clonal but relatively small patches carrying such molecular abnormalities [77]. LOH of chromosomes 2q and 12p is also found in over 10% of normal epithelial samples from lung cancer patients, and like 3p LOH, these losses are significantly associated with smoking [78,79]. LOH of chromosomes 9p and 8p seems less frequent but also occurs early, suggesting it might follow deletions on other chromosomes [80]. Thus, LOH at certain chromosomal regions appears to be a very early event during the development of precancerous fields. Which genes are targeted is still unclear, but observed hypermethylation of loci in the areas of LOH might provide clues (see below).

Interestingly, an analysis by Wistuba et al. of the specific parental allele lost in multiple preneoplastic lesions and squamous cell lung carcinomas from the same patients indicated that the 3p allele loss was non-random [75]. Could such allele-specific events perhaps be linked to DNA methylation changes arising from loss of parental imprinting (LOI)? Global loss of imprinting in a mouse model leads to widespread tumorigenesis in various organs [81]. The imprinted locus H19 has been shown to be monoallelically upregulated (without LOI) in histologically normal lung from smokers [82], and LOI-mediated overexpression of H19 has been found to be frequent in lung cancer [83]. LOI of the MEST and IGF2 genes is also frequent in lung adenocarcinoma [84,85]. Thus, LOI occurs in lung cancer, and could perhaps be related to allele-specific chromosomal losses. However, at this time there is little evidence for fields of LOI in histologically normal lung [84]. Pinpointing the timing of LOI events will require further investigation and may show that LOI occurs at different times for different loci [84].

It has been widely documented that *global* hypomethylation, i.e. the loss of methylation seen at sporadic (non-CpG island) CpGs is seen in many types of cancer. This has been shown to contribute to chromosomal instability, and thus could be a factor in early LOH events. Studies of global hypomethylation in human squamous cell lung cancer showed hypomethylation of the cancers compared to uninvolved bronchial mucosa and hyperplastic epithelium, suggesting that global hypomethylation is not an early event [86]. Mouse experiments with DNA methyltransferase hypomorphs have demonstrated that reducing global methylation severely limits the development colon adenocarcinoma, while promoting lymphomas, cancers that appear to rely heavily on chromosomal rearrangements [87,88]. Based on the parallels between colon adenocarcinoma and lung adenocarcinoma, these animal experiments would suggest that during early stages of cancer de-

velopment, *hyper*methylation is much more important than hypomethylation. This would imply that global demethylation does not underlie early instances of LOH.

What then causes early LOH? Perhaps these local chromosomal losses are repair deficiencies associated with carcinogen-induced damage and possible loss of repair gene function. Genes involved in repair might be mutated or silenced, for example by CpG island hypermethylation. Analysis of adjacent non-tumor lung from lung cancer patients, non-tumor lung from non-cancer patients, and bodily fluids from clinically free cancer patients (sputum, bronchioaveolar lavage, blood) has provided ample evidence for hypermethylation as a very early event in lung cancer development. Table 1, column 3 indicates whether methylation of certain loci has been found in histologically normal lung.

The presence of hypermethylation in tumor margins suggests that altered fields of cells exist in the lung [43]. Substantial evidence supports a role for environmental exposures in promoting such changes. An excellent study of lung biopsies from smokers and non-smokers showed CDKN2A methylation in 18% of non-cancerous biopsies from smokers, but no methylation in biopsies from never-smokers [89], suggesting that epigenetic changes arise in response to environmental insults. In addition, hypermethylation of CDKN2A in lung lobes without cancer was concordant with the primary tumor in almost all cases, suggesting frequent occurrence of methylation of this locus in bronchial epithelium of smokers [89]. Hypermethylation of RARB, CDKN2A, DAPK, RASSF1A and MGMT was observed in the serum of non-cancer cases, and was more frequent in $\geqslant$40 pack-year smokers [90]. Hypermethylation of CDKN2A and MGMT has also been observed in sputum from cancer-free smokers [91–94], and hypermethylation of CDKN2A, RASSF1A, MGMT and APC in non-small cell lung cancer are more frequent with increasing years of smoking [38,39,95,96]. The role of smoking in causing hypermethylation is further supported by the fact that the number of methylated loci (the "methylation index"), is progressively elevated in former smokers and current smokers [39].

Methylation-based silencing could complement LOH, for example of genes on chromosome 3p. Several 3p loci, such as FHIT, RARB, RASSF1, SEMA3B, and TGFBR2 can be methylated to some extent in histologically normal lung (Table 1). SEMA3B, which lies at 3p21.3 and encodes a putative tumor suppressor protein that competes with vascular endothelial growth factor (VEGF) for binding to two neuropilin receptors [97], was shown to be methylated in $\sim$11% of adjacent non-cancerous lung from lung cancer patients, and 50% of lung adenocarcinomas [98]. Like SEMA3B, many loci that show early methylation exhibit increasingly frequent methylation in BAC and adenocarcinoma (Table 1), suggesting that hypermethylation is progressive during lung adenocarcinoma development. Studies with lung cancer-prone mice demonstrated that reduced expression of the DNA methyltransferase DNMT1 resulted in a 50% reduction in tobacco carcinogen-induced pulmonary lesions [99]. The effect was even more pronounced when the mice were simultaneously treated with histone deactelylase inhibitors, emphasizing the importance of epigenetic silencing in lung adenocarcinoma development. Aside from tobacco smoke, components of diesel exhaust have also been shown to increase CDKN2A methylation in rats [100]. Together, these observations support a role for environmental insults such (as smoking) in DNA methylation changes that could be initiated at a time when lung tissue appears histologically normal.

It has been proposed that tobacco smoke could promote epithelial-mesenchymal transition of cells in lung cancer, through its effect on pathways such as WNT, thereby disrupting intracellular communications, promoting cell motility and causing changes in gene expression [101]. Indeed, the WNT pathway has been implicated in self-renewal of stem cells [102]. APC is a well known negative regulator of WNT, that is mutated in familial colon cancer [103]. Because in lung cancer few *mutations* have been found in APC, pathways for WNT activation distinct from the canonical pathways have been proposed [102]. However, hypermethylation of APC is commonly observed in lung cancer as well as in histologically normal lung from older subjects, and thus *epigenetic* silencing of APC could represent a mechanism for WNT pathway activation in the lung [38,50].

A possible confounding factor in the analysis of methylation in non-tumor lung is age-related hypermethylation [27,104]. Analysis of autopsy lung from non-lung cancer patients showed an elevated frequency of methylation in subjects older than 44 years of age, compared to subjects younger than 32 years old [105]. In the younger subjects, only APC was methylated (in about 30% of cases), whereas in the older group, 17% of subjects showed DAPK and CDH1 methylation and 5–6% of cases exhibited methylation of CDKN2A, RASSF1 and/or RUNX3. These observations reinforce the notion that aging alone can lead to hypermethylation, a consideration that should be kept in mind as re-

Table 1
Hypermethylation of loci in histologically normal lung tissue (from non-lung cancer patients or from tissue adjacent to lung cancer) and in lung adenocarcinoma

Locus (HUGO Name)	Locus description [chromosomal location]	Methylated in non-tumor lung[a]	Methylated in adenocarcinoma[b]	References
ACIN1	apoptotic chromatin condensation inducer [14q11.2]	++	More frequently methylated in tumors.	[148]
ADAMTS8	ADAM metallopeptidase with thrombospondin type 1 motif, 8 [11q25]	+	More frequently methylated in tumors.	[191]
APC	adenomatosis polyposis coli [5q21-q22]	+++	More frequently methylated in tumors. More frequently methylated in smokers. Associated with poor survival.	[39,105,192] [38,50,184,193–195]
CALCA	calcitonin/calcitonin-related polypeptide, alpha [11p15.4]	+++	More frequently methylated in tumors.	[50]
CCND2	cyclin D2 [12p13]	−	40% methylated in tumors (NSCLC).	[196]
CDH1	cadherin 1, type 1, E-cadherin (epithelial) [16q22.1]	++	More frequently methylated in tumors. Associated with poor prognosis.	[50,105,195,197]
CDH13	cadherin 13, H-cadherin (heart) [16q24.2]	+	More frequently methylated in tumors. No correlation with smoking. Associated with late stage.	[39, 198–200]
CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)[11p15.5]	−	Methylated in 32% of tumors (NSCLC).	[201]
CDKN2A	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4) [9p21]	+/++	More frequently methylated in tumors. Methylation correlated with smoking and poor survival. Increased frequency of methylation in metastatic lesions.	[38,39,50,90,93, 105,109,115,144, 198,200,202–206]
CHFR	checkpoint with forkhead and ring finger domains [12q24.33]	−	10% methylated in tumors (NSCLC).	[207]
DAB2	disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila) [5p13]	−	30% methylated in tumors.	[208]
DAPK	death-associated protein kinase 1 9q34.1	++	More frequently methylated in tumors. No correlation with smoking. Associated with poor survival, positive nodes and late stage.	[90,105,209–212]
EPB41L3	erythrocyte membrane protein band 4.1-like 3 [18p11.32]	ND	Methylation frequency increases with stage.	[213]
ESR1	estrogen receptor 1 [6q24-q27]	−/+	Frequently methylated in (non)smoking men, in women, predominantly methylated in non-smokers	[50, 214, 215]

Table 1, continued

Locus (HUGO Name)	Locus description [chromosomal location]	Methylated in non-tumor lung[a]	Methylated in adenocarcinoma[b]	References
ESR2	estrogen receptor 2 (ER beta) [14q21-q22]	+	More frequently methylated in tumors.	[50]
FBN2	fibrillin 2 (congenital contractural arachnodactyly) [5q23-q31]	+	More frequently methylated in tumors. Associated with large tumors and nodal metastasis.	[216]
FHIT	fragile histidine triad gene [3p14.2]	+	More frequently methylated in tumors. Methylation correlated with poor survival by some and invasion/lymph node metastasis by others.	[110,197,200, 217–221]
GATA4, 5	GATA binding protein 4, 5 [8p23.1-p22, 20]	−, but present in 50% of BAC	More frequently methylated in tumors.	[222]
GJA1	gap junction protein, alpha 1, 43 kDa (connexin 43) [6q22-q23]	++	More frequently methylated in tumors.	[223]
GSTP1	glutathione S-transferase pi [11q13-qter]	−/+	Not a very frequently methylated locus in tumors.	[39,50,105]
HIC1	hypermethylated in cancer 1 [17p13.3]	++	Methylation frequency increases with decreasing differentiation.	[224]
HTR1B	5-hydroxy-tryptamine (serotonin) receptor 1B [6q13]	ND	Methylated in 6 out of 8 tumors.	[225]
HOX genes	homeobox A , D cluster [7p15-p14, 2q31-q37]	+/++	Depending on which HOX gene, 25–100% methylated in tumors.	[109,226]
IGSF4	immunoglobulin superfamily, member 4 [11q23.2]	+	More frequently methylated in tumors.	[227]
LAMA3,B3	laminin alpha 3, beta 3 [18q11.2, 1q32]	++	More frequently methylated in tumors.	[228]
MGMT	O-6-methylguanine-DNA methyltransferase [10q26]	+/++	More frequently methylated in tumors. One report of association with non-smoking status. More common in larger tumors.	[39,50,90,93,146, 158]
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli) [3p22.3]	−	38% methylated in tumors. Methylation correlated with late stage and poor survival.	[67,105]
MTHFR	5,10-methylenetetrahydrofolate reductase (NADPH) [1p36.3]	+++	More frequently methylated in tumors.	[50]
MYO18B	myosin XVIIIB [22q11.2-12.1]	+	More frequently methylated in tumors. (NSCLC)	[229]
MYOD1	myogenic differentiation 1 [11p15]	++	More frequently methylated in tumors.	[50]
PAX5	paired box gene 5 (B-cell lineage specific activator) [9p13]	+++	50–60% methylated in adenocarcinoma; frequency similar to adjcent non-tumor lung.	[230]
PGR	progesterone receptor [11q22-q23]	++	More frequently methylated in tumors.	[50]

Table 1, continued

Locus (HUGO Name)	Locus description [chromosomal location]	Methylated in non-tumor lung[a]	Methylated in adenocarcinoma[b]	References
PRKCDBP	protein kinase C, delta binding protein [11p15.4]	+	More frequently methylated in tumors.	[231]
PTEN	phosphatase and tensin homolog (mutated in multiple advanced cancers 1) [10q23]	ND	6–24% methylated in adenocarcinoma.	[232,233]
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) [1q25.2-q25.3]	+++	Methylated in 7/7 tumor samples.	[50]
PYCARD	PYD and CARD domain containing [16p12-11.2]	−	More frequently methylated in tumors.	[234]
RARB	retinoic acid receptor, beta [3p24]	+ Methylated in 13–55% sputum of never/heavy smokers.	More frequently methylated in tumors. One report of association with smoking and late stage.	[39,90,198, 200,204,220, 235–237]
RASSF1	Ras association (RalGDS/AF-6) domain family 1 [3p21.3]	−/+	More frequently methylated in tumors. More frequently methylated in smokers. Correlation shown to age at which smoking started. No correlation to stage. One study shows association with poor survival.	[39,105,198, 238] [38,50,90, 96,204,195, 200,220, 239–242]
RPRM	reprimo, TP53 dependent G2 arrest mediator candidate [2q24.1]	+	More frequently methylated in tumors.	[243]
RUNX3	runt-related transcription factor 3 [1p36]	+	More frequently methylated in tumors. Methylation more frequent in non-smokers than smokers.	[105,244]
SCGB3A1	secretoglobin, family 3A, member 1 [5q35-qter]	++	More frequently methylated in tumors.	[245]
SEMA3B	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B [3p21.3]	−/++	More frequently methylated in tumors. No correlation with tumor stage.	[98,238]
STK11	serine/threonine kinase 11 (LKB1) [19p13.3]	ND	Infrequently methylated, more likely mutated.	[130,132]
SOCS3	suppressor of cytokine signaling 3 [17q25.3]	++	More frequently methylated in tumors.	[246]
SPARC	secreted protein, acidic, cysteine-rich (osteonectin) [5q31-q33]	+	More frequently methylated in tumors, and correlated with poor survival.	[247]
TGFBR2	transforming growth factor, beta receptor II (70/80 kDa) [3p22]	++	More frequently methylated in tumors, not correlated with poor survival.	[248]
TIMP3	TIMP metallopeptidase inhibitor 3 (Sorsby fundus dystrophy, pseudoinflammatory) [22q12.1-q13.2]	++	More frequently methylated in tumors.	[50]
TNFRSF10C,D	tumor necrosis factor receptor superfamily, member 10C, D. Decoy without	−	12–15% methylated in tumors.	[249]

Table 1, continued

Locus (HUGO Name)	Locus description [chromosomal location]	Methylated in non-tumor lung[a]	Methylated in adenocarcinoma[b]	References
	an intracellular domain [8p22-p21]			
WIF1	WNT inhibitory factor 1 [12q14.2]	++	More frequently methylated in tumors.	[250]
WWOX	WW domain containing oxidoreductase [16q23.3-q24.1]	+	Frequently methylated in squamous cell cancer, adeno was not examined.	[217]
ZMYND10	zinc finger, MYND-type containing 10 [3p21.3]	−	30% methylated in tumors. No correlation with stage.	[238]

[a]Approximate frequencies of methylation in histologically normal lung are indicated as follows: + present but infrequent (<10%); ++ moderately frequent (∼10–25%); frequent (>25%).
[b]Data given refers to lung adenocarcinoma, unless otherwise indicated.

searchers try to identify methylation caused by environmental influences such as smoking. Using the sensitive and quantitative MethyLight technology, we have also found substantial methylation in histologically normal lung from patients operated for non-cancer reasons, such as lung collapse or emphysema [50]. Low levels of APC methylation were found in 9/11 non-tumor lung samples from non-lung cancer patients, while elevated MTHFR methylation was found for almost all non-tumor lung and adenocarcinoma samples. RASSF1 showed modest methylation in 50% of non-tumor lung samples. CDKN2A methylation was found in 8/11 non-tumor lung samples. In the absence of information on smoking, it is unclear whether the observed methylation in non-tumor lung could be environmentally induced or simply is a consequence of age-dependent methylation. However, the very low levels of APC methylation observed in mesothelioma suggest that if age is crucial, its effects vary per tissue type. Indeed, in the autopsy analysis of different tissues, APC was differentially methylated in different parts of the digestive tract [105]. It is conceivable that age-related methylation plays a part in cancer development, perhaps as an added risk factor. Indeed, many lung cancer patients are over age 65. In the case of young, non-smoking adenocarcinoma patients, clearly other mechanisms must be at work.

Given the early abundance of *hyper*methylation in preneoplastic lung, one could wonder whether there is any evidence for early *de*methylation of CpG islands. While hypomethylation of certain genes has been observed (see below), it appears to be much less common than hypermethylation, which has been shown to occur widely at CpG islands throughout the genome [28, 29]. A prominent example of cancer-specific local demethylation is the reactivation of so-called cancer testes (CT) antigens. These are genes that are normally only expressed in the human germline, but become active in certain cancers, such as melanoma, bladder, liver and lung cancer (reviewed in [106]). Some CT genes are X-linked, and others are spread over the autosomal chromosomes. Aberrant expression of these genes frequently coincides with loss of CpG island methylation [106]. In an immunohistochemistry study of 12 CT antigens in lung cancer, 68% of adenocarcinomas showed expression of one or more gene, but no expression was evident in adjacent histologically normal lung [107]. However, a different study found hypomethylation and expression of CT antigens in up to half of the normal lung samples adjacent to tumors, and up to 60% of normal bronchial epithelium from previous smokers [108]. The discrepancy between these two studies might arise from the use of the more sensitive RT-PCR approach in the latter study. In support of the early activation of CT antigen genes, a study of sputum showed hypomethylation of the MAGE CT antigens in up to 24% of cytologically normal samples [109]. Thus CT antigen re-expression appears to be a very early event that may play a role in field defects. It might provide cells with properties that aid dedifferentiation, proliferation or survival [106].

In conclusion, it appears that many (epi)genetic alterations are found in non-cancer lung (Fig. 2). Some of these changes may be associated with aging, while others are likely consequences of environmental exposures. Irrespective of their etiology, such changes could promote lung cancer development. LOH and CpG island hypermethylation appear to be common events that may even target the same gene at different times. For example, FHIT, a candidate tumor suppressor seen to be lost in *tumors* through LOH at 3p14.2, does not show LOH in AAH. However, this does not mean the gene is not inactivated at an early stage; methylation of this locus is seen in lavage fluid from cancer-negative

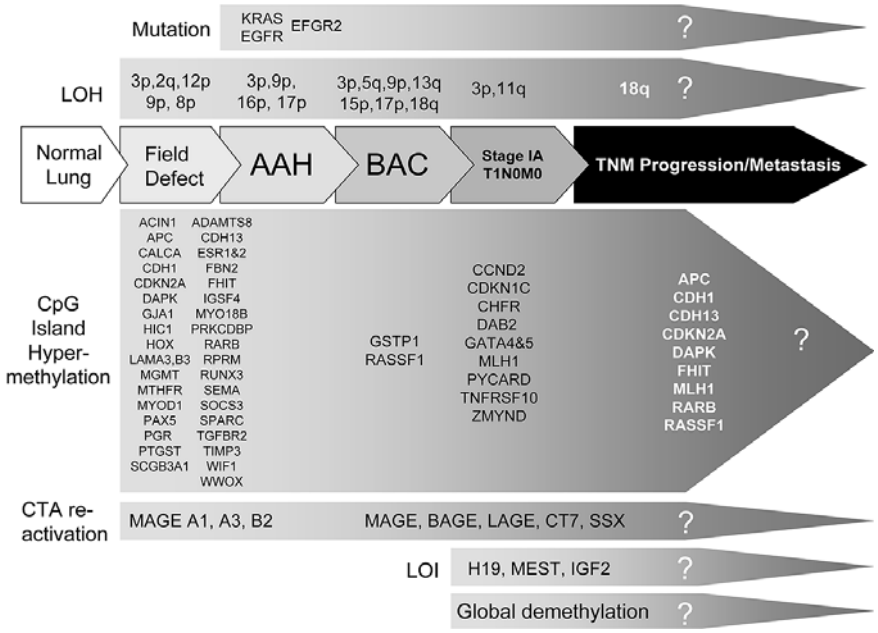

Fig. 2. A framework for sequential (epi)genetic changes during development and progression of lung adenocarcinoma. The progressive stages of lung adenocarcinoma development are indicated by a schematic consisting of overlapping arrows at the center. A partial list of genetic alterations (LOH and mutations) are indicated above, and epigenetic alterations are marked below this schematic. (Epi)genetic lesions are marked above or below the approximate stage of cancer development where they are first seen. It should be noted that this list does not aim to be complete, but is intended to show the diversity of changes and the fact that many changes occur early during adenocarcinoma development. Hypermethylated loci are listed only once in the black text area, even if the methylation frequency increases at later times. The identity of many (epi)genetic alterations remains to be discovered, as indicated by the question marks. Changes known to affect progression (late stage, large tumor size, metastasis and/or poor survival) are marked in white. Loci for which hypermethylation is associated with progression are listed to the right in white text.

cases [110]. Expression of CT antigens is also an early event seen in histologically normal lung. This might lead to alterations in proliferation and differentiation, perhaps providing cells with cancer stem cell-like properties. Loss of growth control would be further supported by decreased expression of cell cycle regulator CDKN2A, whose methylation appears to be a very early event. Decreased CDKN2A expression would bypass the need for mutations of RB1 (which functions downstream). Indeed, RB1 mutations are infrequently seen in lung adenocarcinoma [111,112] and seem to be mutually exclusive with CDKN2A alterations [113–115]. Age- or environmentally-induced APC methylation could lead to activation of the WNT pathway and further stimulation of growth through induction of genes such as MYC. Together, these changes would set the stage for the development of foci of AAH. Clearly, much remains to be learned about changes in histologically normal lung, and it would be of great interest to establish detailed methylation profiles from a large number of cases, to compare the results of methylation profiles in tumors and adjacent non-tumor lung from the same subjects, and to correlate methylation levels to environmental exposures. Further studies of lung from non-cancer subjects with varying degrees of environmental exposures would also contribute importantly to insights into the role of aging and the environment. Detailed insights into the molecular changes seen in histologically normal lung will be of great use for the development of chemoprevention strategies. Given the prominent presence of hypermethylation early in the process of adenocarcinoma development, delivery of chemopreventive methylation inhibitors may be one avenue of clinical research worth exploring.

5. (Epi)genetic changes in AAH

Immunological characterization of AAH shows increased staining of TP53, cyclin D1 (CCND1) and the anti-apoptotic protein Survivin (BIRC5), and a decrease in CDKN2A and CDKN1B expression [51,116–118]. However, immunological analysis does not shed light on the mechanism by which such aberrant expression is achieved. Molecular examination of AAH at the level of DNA is challenging due to the limited

size of these lesions, which are generally smaller than 5 mm and contain few cells due to their alveolar structure [51]. For this reason, the (epi)genetic analysis of AAH is still quite limited. Most studies to date have focused on examining LOH and a limited set of mutations in proto-oncogenes and tumor suppressors [51]. To our knowledge, no studies of DNA methylation in AAH have been published.

Based on the occurrence of LOH in histologically normal lung one might expect overlapping and progressive losses in AAH. Indeed, when compared to histologically normal adjacent lung, AAH shows LOH of distinct regions of chromosomes 3p, 9p, 16p and 17p [119–121]. Loss of 9p was found in AAH in 7–33% of cases, and in some reports became more frequent as the lesions progressed to adenocarcinoma [119,122]. As in adjacent non-tumor lung (above) these losses are thought to happen sequentially, targeting distinct regions at different times. For example, FHIT, although located in a chromosomal region commonly showing LOH (3p14.2), does not appear to be deleted in AAH; LOH is not evident until BAC and adenocarcinoma [123,124].

Interestingly, in over half of the cases of LOH in AAH, the 9p region included the TSC1 locus [121]. In addition, 22% of AAH showed loss of 16p, and 6% of these were in 16p13.3, the location of TSC2. The TSC genes are of substantial interest because their proteins form a negative regulatory complex in the PI3K pathway, which mediates signals from growth factor/hormone/cytokine receptors to the FRAP1 (mTOR) pathway. FRAP1 plays a powerful role in the control of cell growth by modulating the levels of mRNA translation and proliferation [125,126]. Inherited mutations of TSC1 cause mental retardation and a host of other effects, including AAH-like lesions in the lung. The link between TSC genes and AAH is further strengthened by the observation that adenocarcinomas showing LOH at TSC1 or TSC2 frequently show AAH [127]. A search for mutations in the two TSC genes in AAH showed these were uncommon [128], but to our knowledge, hypermethylation of TSC1 and 2 promoter CpG islands, which can occur in breast cancer [129], has not been examined in lung cancer. Another gene in the FRAP1 pathway is tumor suppressor LKB1, which functions upstream of TSC1/TSC2. Germline mutations in LKB1 predispose to a variety of cancers, including occasional lung adenocarcinomas. Mutations in LKB1 have been found in 30% of sporadic adenocarcinoma [130], and loss of LKB expression is seen 5% of low grade AAH, and 21% of high grade AAH [131]. Methylation of LKB1, which is infrequent in adenocarcinoma [132], is not the mechanism for LKB1 inactivation. Other changes are also seen in the translational regulatory pathway during the evolution of normal cells to AAH, BAC, and beyond, such as a progressive increase in eukaryotic initiation factor 4E (EIF4E) [133]. Thus, increased translation appears to be a hallmark of developing adenocarcinoma.

As the cells that make up AAH lesions increase their translation and thus their protein output, it seems likely that their cell cycle would also be deregulated. A decrease in CDKN2A expression has been observed in AAH [118]. CDKN2A already shows methylation in histologically normal lung (Table 1), but does methylation increase in AAH to further reduce cell cycle control? We have recently carried out a study of four cases, comparing CDKN2A methylation in AAH with matched associated adenocarcinoma and non-tumor lung. Preliminary data obtained using the quantitative methylation assay MethyLight [134,135] showed that, as expected, CDKN2A was somewhat methylated in all four samples of histologically normal lung. In three out of four matched AAH lesions and in all matched adenocarcinoma samples, the level of methylation was more than twice as high (Galler, Kerr and Laird-Offringa, unpublished results). This suggests that further silencing of CDKN2A occurs as lung cells progress to AAH lesions.

In addition to molecular changes that promote cell cycle transitions, changes affecting the response to growth signals might also be early events that occur in AAH. RAS proteins play a role in mediating receptor tyrosine kinase signaling. KRAS mutations are found in 14–30% of lung adenocarcinomas [136–138]. Such mutations have also been observed in AAH lesions [55, 56,139]. In support of the idea that KRAS activation is an early event, it was noted that these mutations are present in adenocarcinomas from longtime smokers as well as tumors from patients who had quit smoking many years ago, but are absent in adenocarcinomas from non-smokers [136].

Activation of the receptor tyrosine kinase signal transduction pathway is one of the growth stimulatory changes seen in lung adenocarcinoma, but KRAS mutations are only found in a limited percentage of cases. This suggests that alternative molecular changes might be found in cells lacking activated RAS. One strong candidate is mutation of the epidermal growth factor receptor (EGFR), a gene activation event that has received much recent publicity because it sensitizes lung cancers to treatment with certain tyrosine kinase in-

hibitors (reviewed in [138]). Tyrosine kinase receptors signal through RAS, so that the action of these two genes converges. Interestingly, EGFR mutations appear to be more common in the lung cancers of Asian non-smoking women, the same group that shows a link with HPV infection. As would be expected, EGFR and KRAS mutations appear to be mutually exclusive [138]. A recent examination of the methylation status of 5 genes and EGFR and KRAS mutations [140] confirmed this observation, and in addition, showed an inverse relationship between EGFR mutations and methylation of CDKN2A and CHD13 (adhesion molecule H-cadherin). What is the status of EGFR mutations in AAH? The reported percentage of lesions with such a change varies from 3 to 40% [141,142]. This variability may be related to differences in etiology of the examined samples; AAH from the lungs of non-smokers exposed to HPV would be much more likely to show EGFR mutations, and lack CDKN2A methylation since HPV protein E7 inactivates RB1 [143]. Based on the previously shown association, CDH13 methylation may also be less likely in these patients, though the mechanistic link to EGFR mutations is not yet clear. In contrast, smokers would be more likely to show KRAS activation and CDKN2A methylation [140]. If true, this would suggest that there may be at least two parallel molecular pathways to the initiation of lung adenocarcinoma. However, whether CDKN2A methylation is associated with KRAS mutations is controversial [140,144,145]. It will be interesting to determine whether there are epigenetic changes that deregulate the RAS pathway. Inactivation of the mismatch repair gene MGMT might indirectly facilitate the acquisition of RAS mutations in certain types of cancer. Although one report found that MGMT methylation is relatively infrequent in early lung adenocarcinoma lesions [146], we found methylation of MGMT in the cancer-free lung of 8/10 patients operated for non-cancer reasons, and in 7/7 lung adenocarcinomas [50]. Early MGMT inactivation could markedly promote the acquisition of mutations.

In summary, a limited set of molecular changes in AAH have been characterized (Fig. 2). A number of these changes consist of progression of alterations already visible in non-tumor lung, such as further LOH and methylation of CDKN2A. Certain changes are new, such as mutations in KRAS and EGFR. For many of the well characterized (epi)genetic changes observed in adenocarcinoma, it remains unclear whether they occur in AAH or not. The challenge in studying these lesions is their limited availability and their very small size. While immunological staining is feasible and offers detailed insight into molecular alterations as they relate to morphology, this approach requires individual sections for each analysis. The isolation of DNA from microdissected lesions might provide a higher yield of molecular information, potentially allowing the analysis of several loci per section. A very interesting alternative approach to the identification of AAH-specific (epi)genetic alterations was pioneered by Shimada and colleagues, who compared a cell line derived from an AAH lesion (PL16T) with its normal counterpart (PL16B) [147]. A screen for hypermethylated loci yielded ACIN1, apoptotic chromatin condensation inducer 1, which was differentially methylated between the two cell lines [148]. Examination of 37 tumors and paired adjacent non-tumor control tissue showed that ACIN1 was more frequently methylated in BAC/adenocarcinomas than in histologically normal tissue. Further examination of (epi)genetic differences between the two cell lines could provide additional clues to AAH-specific deregulatory events, but this approach would be more powerful if additional AAH-based cell lines were established. A concern is whether these cells will maintain their AAH characteristics. As we await more data from the cell line approach, the collection and analysis of numerous AAH lesions, with analysis of a limited number of markers at a time, will provide slow but progressive insight into (epi)genetic alterations that occur during this preneoplastic stage of adenocarcinoma development. Such findings will be of high relevance for the development of early detection and chemoprevention strategies.

6. (Epi)genetic changes during progression from AAH to adenocarcinoma

As AAH lesions grow and become more atypical, losing their characteristic structure, they become indistinguishable from BAC [149]. Changes in cell-cell contacts are illustrated by incremental loss of expression of CDH1 (encoding E-cadherin, a membrane glycoprotein thought to play a role in adhesion). CDH1 is expressed in AAH but begins to decline in BAC, with further loss of expression in adenocarcinoma [150]. Matrix metallopeptidases (MMPs) become elevated in the BAC part of mixed adenocarcinoma/BAC lesions [151], hinting at the acquisition of invasive properties. A gradual increase in proliferation markers, such as components of the telomere complex and translation initiation factor eIF4E is seen as lesions progress

from AAH to BAC, and then to adenocarcinoma [62, 133,152]. These observations indicate that changes associated with growth deregulation can progress as the lesion develops from high grade AAH into BAC. LOH continues to become more evident. An analysis of adenocarcinomas with BAC component (used because true non-invasive BAC is rare) showed frequent 5q and 17p losses in what would be considered the lowest grade sections of the tumors [153]. A detailed investigation of loci associated with LOH, using BACs and small adenocarcinomas, showed localized loss of 5q(APC), 9p(CDKN2A), 13q(RB1), 17p(TP53) and 18q(SMAD4) in BAC, while LOH of 3p(FHIT) and 11q(INT2) did not become prominent until invasion [154], consistent with the observed persistence of FHIT protein expression in AAH and BAC but loss of expression in invasive disease [124]. The late loss of FHIT is interesting in light of the observation of methylation of this locus in lavage fluid from cancer-negative cases [110], hinting at the potential for genes to become inactivated by different mechanisms at different times. In the case of CDKN2A, biallelic inactivation through LOH combined with hypermethylation was seen in 22% of adenocarcinomas, providing a mechanism for progressive CDKN2A deregulation [155].

One alteration seen in high grade AAH/BAC is EGFR2/HER2 expression [156]. Like EGFR mutations, mutations in EGFR2 are found most commonly in adenocarcinoma from female Asian non-smokers, and are mutually exclusive with KRAS alterations [138, 157]. The observed elevated EGFR2 expression in AAH/BAC suggests that the tyrosine kinase signaling pathway is altered by a variety of mechanisms before adenocarcinoma reaches its invasive stage. The methylation of DNA repair gene MGMT, which is seen in histologically normal lung but increases in frequency to 28% in pure BAC, could potentially facilitate the acquisition of further genetic hits in this and other pathways [158]. As can be seen in Table 1, many other loci that show some frequency of methylation in histologically normal lung adjacent to adenocarcinoma show more frequent methylation as adenocarcinoma develops. This includes genes involved in the cell cycle, DNA repair, differentiation, cell-cell contacts and adhesion, and signaling. In addition, loci for which methylation in histologically normal lung has not been seen, such as cell cycle control genes CCND2 and CHFR, signaling molecules DAB2 and TNFRSF10 C and D (trail decoy receptors), transcription factors GATA4 and 5, and DNA repair gene MLH1, now show methylation (Fig. 2). The widespread methylation observed in lung adenocarcinoma speaks to the crucial role epigenetic alterations play in the development and progression of this disease. Considering that only a very small fraction of the approximately 30,000 CpG islands in the genome [159] has been examined, much further information remains to be gathered. The use of high throughput and epigenomic approaches will greatly accelerate the accrual of information about epigenetic alterations in lung adenocarcinoma [35,134, 135,160]. Among a variety of new approaches, the use of CpG island microarrays is considered to be one of the most powerful ones; it can be used to examine the methylation status of thousands of genes simultaneously [160]. With the application of this and other techniques, rapid progress in the identification of DNA methylation changes can be expected in the coming years.

7. (Epi)genetic changes during progression to metastasis

It is usually the metastatic spread of cancer, not the primary lesion, that is the cause of cancer death. Thus, the investigation of (epi)genetic alterations that enable metastasis is crucially important. Successful metastasis not only depends on the ability of cancer cells to escape from the initial site, but also to survive and proliferate in the new location [161,162]. Evidence indicates that the local environment has a strong influence on the ability of metastatic cells to grow. It has been proposed that micrometastases might lie dormant until particular metastasis-suppressing genes are lost or inactivated [161,162]. Whether this is true for lung cancer remains a question, though some data in support of this idea has been provided. A recent examination of the prognostic significance of lung cancer patient nodes carrying either isolated tumor cells, micrometastases (< 0.2 mm), or lesions $\geqslant 0.2$ mm showed that patients with nodes positive for isolated tumor cells or micrometastases did not exhibit a shorter survival than node-negative cases [163]. The number of cases examined in the study were relatively small (60 patients), so that the results should be interpreted with caution, but the observations hint at the potential requirement for additional genetic and/or epigenetic changes to allow metastases to flourish in their new location. These metastasis-specific molecular changes could consist of the inactivation of metastasis suppressor genes or the activation of genes that promote proliferation. There are at least two striking examples of genes that un-

dergo opposing alterations to promote both metastasis and proliferation. One example is CD44, encoding the hyaluronic acid receptor, a protein that mediates cell-cell and cell-matrix interactions [164]. Increased expression of CD44 promotes metastasis, while reduced expression promotes local growth. The inherent reversibility of DNA methylation makes this mechanism of gene (in)activation particularly suited for inversions of gene deregulation during metastasis. A second example is provided by CDH1. Increased methylation and reduced expression of CDH1 favors metastasis, while reduced methylation and increased expression favors growth [165,166]. These examples illustrate that the role of DNA methylation in metastasis can be highly complex.

What is known about methylation-based (in)activation in lung adenocarcinoma to date? The frequency of methylation of several loci is linked to progression (Table 1, Fig. 2). Hypermethylation of APC, CDH1, CDH13, CDKN2A, DAPK, FHIT, MLH1, RARB, and RASSF1 in adenocarcinoma has been associated with larger tumor size, late stage, poor survival, and/or nodal metastases (Table 1). Indeed, the number of methylated genes in a given tumor sample has been shown to be correlated with the presence of brain metastases from primary melanomas, breast cancers and lung carcinomas [167]. In addition to methylation of the well known genes listed above, it is likely that the inactivation of previously unmethylated loci encoding metastasis suppressor genes will play a role in the ability of tumors to spread to secondary sites. Metastasis suppressor genes have been identified in different kinds of cancer [161, 162] and are defined as genes that "encode proteins that specifically suppress metastasis formation while having no discernable effect on primary tumor growth" [162]. Examples are osteoblast cadherin (CDH11 [168]), non-metastatic gene family members (eg. nm23 genes such as NME2 [162]), and ribonucleotide reductase M1 subunit (RRM1 [169]). To our knowledge, none of these markers have been evaluated for hypermethylation in lung cancer.

Just as inactivation of a variety of genes could promote metastasis, increased expression of others could be required. This can be achieved through epigenetic mechanisms, as illustrated by the observation that treatment of cancer cells with the DNA methylation inhibitor 5-azacytidine promotes micrometastais formation [170,171]. Hypomethylation of the neuronal synuclein-gamma gene is linked to metastasis of diverse human tumors, including lung cancer [172]. Re-expression of cancer testes antigens could also contribute in a variety of ways to metastatic potential [106]. Understanding the mechanistic complex of methylation to metastasis will require extensive further investigation.

8. DNA methylation as a marker for lung adenocarcinoma diagnosis and prognosis

While it will take many more years before the mechanistic roles of (epi)genetic changes in lung adenocarcinoma development and progression are understood, in the short term, simply cataloging such changes could provide valuable diagnostic and staging tools. Genetic alterations can be just as informative as epigenetic ones, however, the analysis of promoter CpG island hypo/hypermethylation as a cancer marker has several important advantages. The first is that the alteration is clearly localized. Mutations that activate or inactivate proteins can be located in many different regions of a gene (with the exception of certain cases in which very specific amino acids are altered, such as the RAS genes), necessitating the examination of large sections of sequence. In contrast, the CpG islands of interest largely lie near the transcription start site of genes. This allows the rapid genome-based design of probes and primers that can provide accurate measures of methylation for any locus of interest. A second advantage is that methylation alterations are not an all or nothing phenomenon. They can be present to variable degrees and with variable frequencies for different loci. Thus, they can provide a richness of information about a locus that is unparalleled, except perhaps by gene expression analysis. Quantitative methylation analysis can be used to compare levels of methylation in different tissues or in similar tissues under different conditions [135]. Thirdly, DNA methylation information is robust; it is well maintained in archival specimens. Although prolonged storage of archival specimens can result in severe fragmentation of DNA, using MethyLight [134], we have been very successful in obtaining methylation information from paraffin-embedded material that was over ten years old (Laird-Offringa laboratory, unpublished data). DNA methylation information is also relatively stable in biological fluids from patients. Many studies have demonstrated that methylation data can be reliably obtained from a variety of bodily fluids, including sputum, serum, urine, and stool [30,173,174]. Thus, analysis of DNA methylation can be used for retrospective studies using archival collections, as well as for the examination of fresh biological specimens

from cancer patients. Fourth, DNA methylation can be very sensitively detected, either by real-time PCR or nested PCR of bisulfite-treated DNA. (Bisulfite treatment incorporates the methylation information into the DNA sequence, by converting unmethylated Cs to U, while leaving methylated Cs intact [175]). Fifth, the availability of ∼30,000 CpG islands allows the development of many different markers that can be simultaneously assessed on the same platform. Thus, methylation markers can be easily combined into panels that could increase sensitivity and specificity.

One key consideration in the application of DNA methylation analysis as a marker for cancer is the fact that, like any biomarker, it is used for a comparative analysis. The choice of compared tissues must be carefully weighed to provide information relevant to the intended application. Such applications could be cancer detection, disease monitoring, cancer staging, detection of premalignant lesions, cancer classification, or prognostication. One very promising application of methylation markers is their use as a complement to low-dose spiral computed tomography (LDSCT), which can provide detailed images of the lung. LDSCT has been advocated for the screening of subjects at risk for lung cancer (long term heavy smokers), and several pilot studies have explored its potential for early detection [176,177]. However, LDSCT detects pulmonary nodules in up to 50% of subjects, less than 5% of which will prove to be malignant, and thus could result in overdiagnosis [176,178,179]. Visual interpretation of LDSCT scans is improving, in particular in Japan where screening has been advocated for some time [180], but its accuracy remains limited. Resections are invasive procedures that should be carried out only when they clearly benefit the patient (usually limited to NSCLC stages I, II and occasionally IIIA) [178]. Fine needle aspirates may be used to obtain tumor material for diagnosis, but yields may be low and the reliability of the obtained sample is highly dependent on the ability to accurately locate the biopsy area. Great benefit could be derived from the availability of non-invasively obtainable molecular markers. To this end, methylation signatures could be detected in exfoliative material (sputum, bronchioalveolar lavage, bronchial brushings) or in plasma or serum [31]. It has been demonstrated that tumor DNA from lung cancer patients can be found in exfoliative material [91–94,181] and in the blood of a substantial fraction of cancer patients [182–184]. Because they are DNA-based, methylation markers can be amplified by PCR, and thus provide considerable sensitivity, in particular when quantitative methods are used [31,94,135,160]. However, before methylation markers can be used to complement LDSCT, markers or marker panels with high sensitivity and specificity must be identified.

For the identification of the type of strictly cancer-specific methylation markers that could be used to complement visual lung cancer screening by spiral CT, one would initially compare tumor tissue with histologically normal adjacent tissue from the same patient. This exquisitely controls for environmental exposures and individual genetic variation. Differentially methylated loci identified in this manner are strong candidates for cancer-specific markers. Promising loci could then be further evaluated by comparing methylation levels in patients' bodily fluids (serum, plasma, bronchioalveolar lavage, sputum, exhaled air condensate) to those in similar fluids from subjects with similar exposures but without cancer. This would provide an indication of their clinical sensitivity and specificity. In the case of lung cancer methylation markers, ideally one would identify markers that are specific for every histological subtype of lung cancer, and that can also distinguish between primary lung cancer and metastases to the lung from primary cancers elsewhere in the body. This would allow visually detected lung cancer lesions to be identified not only as primary lung cancer but potentially also as a particular histological subtype.

To develop markers for the detection of precancerous alterations, DNA methylation from a variety of biological specimens (histologically normal, premalignant or cancerous lung, bodily fluids) would be compared between subjects with and without cancer, and differing in their environmental exposures. This approach has been carried out by many, and has provided cancer risk indicators [30,31,94]. An example of an exciting recent study is the examination of 14 loci in the sputum of a high-risk lung cancer cohort. Six loci (CDKN2A, DAPK, GATA5, MGMT, PAX5 and RASSF1) were associated with an increased cancer risk, and methylation of three or more of these genes showed a 65% sensitivity and specificity for lung cancer [94]. Although sensitivity and specificity are not yet optimal, studies such as this one provide an indication of the strong potential of DNA methylation markers for lung cancer detection and risk assessment.

Obtaining lung biopsy samples from healthy individuals is challenging, so that focusing on other types of samples that are obtained through less invasive means would be useful. Bronchioalveolar lavage and sputum are an option (although non-smokers will be less able to provide any sputum), but blood (serum or plasma)

might be a better choice. We are not aware of attempts to measure DNA methylation in exhaled breath condensate, but this may also be worth exploring. By using markers for precancerous lesions to screen at-risk subjects, individuals identified as high risk could be followed more closely, or could be subjected to chemoprevention strategies as they become available. If they are smokers, they should of course be strongly encouraged to stop smoking.

Development of methylation markers as prognostic indicators for survival of lung cancer patients, or for prediction of response to therapy, will require the analysis of DNA methylation profiles of large cohorts of patients and comparison of their clinical variables. To date, while some correlations to prognosis have been found (see Table 1), it would appear that the optimal prognostic markers have not yet been uncovered. Epigenomic comparisons of primary tumors with their corresponding metastatic lesions may be a powerful way to identify node-specific metastasis markers. Such markers would also be useful for staging, through the molecular analysis of sampled nodes. A recent study of node micrometastases in NSCLC patients using promoter hypermethylation assays from fine needle aspirates showed an increase in sensitivity but a loss of specificity when the molecular data was added to conventional cytology assays [185]. More nodes were scored as positive, but these nodes did not necessarily lead to progression, as measured by clinical outcome [185]. This could happen if the markers used were not metastasis-specific. Tumor-specific markers would also identify potentially dormant micrometastases, and thus would reduce specificity, whereas markers that occur only in successful metatstatic growth (in nodes or elsewhere) would provide true metastatic indicators. Besides node-specific metastatic indicators, it should be possible to identify metastatic markers that are present in the primary tumor. Obtaining such markers is more challenging, as tumors that metastasize need to be compared with tumors that do not. Since they are not obtained from the same patient, these comparisons are not optimally controlled for genetic variation and different environmental exposures (in contrast to comparisons between primary tumors and metastases from a single patient). Once such markers are obtained, they could be applied to accurate staging, as a substantial fraction of early stage adenocarcinoma patients (T1N0 or T2N0) later relapse with the development of nodal and / or visceral metastases [186–190].

9. Conclusion

From the sections above, it should be apparent that while much information has been gained, we are still at the very beginning of understanding the mechanistic implications of DNA methylation alterations in lung cancer, of cataloging these changes, and of applying them to the clinic. The advent of high throughput and epigenomic approaches promises rapid progress in the identification of methylation changes. However, the evaluation of the mechanistic and clinical implications of such changes will take many years. This is an incredibly exciting time to study DNA methylation in lung cancer, as it will be possible to make huge strides forward in many areas. Our understanding of lung cancer etiology will be aided by the identification of genes activated or silenced by methylation changes, and by exploring correlations with environmental factors such as smoking and viral infection. DNA methylation markers for diagnosis, classification and prognostication could be tremendously beneficial in the clinic, and could be applied to early detection, the determination of treatment strategies and to the assessment of treatment efficacy. When properly applied, DNA methylation markers could save thousand of lives in the foreseeable future.

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