

Multistep Progression from Atypical Adenomatous Hyperplasia to Lung Adenocarcinoma: Clinico-Pathologic, Epigenetic and Genetic Aspects

Keywords: Atypical adenomatous hyperplasia; Adenocarcinoma *in situ*; Invasive adenocarcinoma; Ground-glass opacity; Molecular alterations

Abstract

Detection of small peripheral ground-glass opacity nodules has increased due to the advances in imaging modalities and the widespread use of computed tomography screening. Pathologic examination of these nodules revealed that they have a pure lepidic or replacement growth pattern such as atypical adenomatous hyperplasia or adenocarcinoma *in situ* (formerly known as bronchioloalveolar carcinoma). When untreated, ground-glass opacity nodules gradually develop a solid component. The greater the solid component or the invasive component, the less favorable outcomes after treatment for patients with ground-glass opacity nodules. Based on the clinical, radiologic and pathologic findings, the concept of multistep progression from preinvasive atypical adenomatous hyperplasia through noninvasive adenocarcinoma *in situ* to invasive adenocarcinoma has been postulated. Recently, evidence has accumulated explaining this putative concept by molecular alterations, including activating mutation of oncogenes and inactivation of tumor suppressor genes by epigenetic changes or loss of heterozygosity. This review 1) comprehensively outlines the accumulated knowledge regarding radiologic and pathologic features of adenocarcinoma and its precursor which presents as ground-glass opacity and 2) summarizes the molecular basis of the multistep progression to lung adenocarcinoma. As a result, we believe identification of undiscovered molecular markers involved in the progression of lung adenocarcinoma is critical for early detection of lung cancer and the development of targeted therapeutic and chemoprevention strategies.

Abbreviations

AAH: Atypical Adenomatous Hyperplasia; ADC: Adenocarcinoma; AIS: Adenocarcinoma *in situ*; BAC: Bronchioloalveolar Carcinoma; CT: Computed Tomography; GGO: Ground-Glass Opacity; LOH: Loss of Heterozygosity; MIA: Minimally Invasive Adenocarcinoma; NGS: Next Generation Sequencing; NSCLC: Non-Small Cell Lung Cancer; PET: Positron Emission Tomography; WHO: World Health Organization

Introduction

Lung cancer is the leading cause of cancer deaths in the United States and worldwide with over 1.3 million deaths in 2008 [1-3]. Despite the fact that enormous resources have been spent on research

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Review Article



Journal of Surgery

Hong Kwan Kim^{1,3}, David M. Jablons^{1,2*} and Il-Jin Kim^{1,2*}

¹Thoracic Oncology Laboratory, Department of Surgery, University of California San Francisco, San Francisco, CA, USA

²Comprehensive Cancer Center, University of California San Francisco, San Francisco, CA, USA

³Department of Thoracic and Cardiovascular Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea

Address for Correspondence

David M. Jablons, MD, Thoracic Oncology Laboratory, Department of Surgery, University of California San Francisco, San Francisco, CA, USA, E-mail: David.Jablons@ucsfmedctr.org

Il-Jin Kim, PhD, Thoracic Oncology Laboratory, Department of Surgery, University of California San Francisco, San Francisco, CA, USA, E-mail: kimij@cc.ucsf.edu

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Submission: 03 July 2013

Accepted: 07 August 2013

Published: 12 August 2013

involving molecular and therapeutic aspects of lung adenocarcinoma (ADC), there has been no significant improvement in the mortality associated with lung cancer for the past 25 years. This can be attributed in part to untimely diagnosis at advanced stages or recurrence occurring even after optimal treatment at early stages. About 70% of patients are diagnosed with lung cancer at advanced stages when there is little chance to cure [4]. Although patients diagnosed at early stages receive curative-intent complete resection by surgery, about 20% of them will not survive due to recurrence within 5 years [5-12]. One cause may be that patients already have microscopic systemic metastases in other distant organs at the time of surgery. In order to reduce the mortality and eventually to overcome lung cancer, understanding carcinogenesis and tumor progression is paramount.

ADC is the most common histologic type of non-small cell lung cancer (NSCLC) in the United States, accounting for almost half of all lung cancers [13,14]. ADC tends to develop distant organ metastases easily even in early stage compared to squamous cell carcinoma, underscoring the need for early detection methods. A multistep progression concept from a precursor lesion (atypical adenomatous hyperplasia, AAH) through noninvasive tumor (bronchioloalveolar carcinoma, BAC; BAC is currently renamed adenocarcinoma *in situ* (AIS), but the term BAC from published articles remained unchanged in this review) to invasive ADC has been postulated based on a variety of clinical, pathologic, and molecular studies [15-18]. It was reported that AAH was frequently detected at the periphery of invasive ADC in surgically resected lungs for pulmonary carcinoma [17,19]. Other reports showed the multistep progression of lung ADC with ground-glass opacity (GGO) features by performing a long-term follow-up with regular CT scans in the same patient [20,21]. This concept appears to be consistent with radiologic-pathologic features and molecular events.

A clear understanding of biological events during the progression of lung ADC will be critical for identifying molecular biomarkers related to carcinogenesis and tumor progression. Ultimately, it will

facilitate early detection of lung cancer, contribute the development of targeted therapeutic strategies, and ideally widen the scope of chemoprevention. This review comprehensively summarizes the accumulated knowledge regarding (1) radiologic features of the preinvasive lesion or early-stage ADC, (2) pathologic findings of AAH, BAC (AIS) or ADC, and (3) genetic and (4) epigenetic alterations associated with the concept of multistep progression of ADC to provide a better understanding of carcinogenesis in a subset of lung ADC.

Radiologic and Pathologic Features

With recent advances in diagnostic imaging modalities, small indeterminate nodules such as GGO lesions have been increasingly detected on high-resolution computed tomography (CT) scans [22-25]. In addition, the introduction of low-dose helical CT scanning for lung cancer screening has further increased the detection rate of small GGO lesions. Recently, the observation from the National Lung Screening Trial that low-dose CT screening can reduce mortality from lung cancer obviously justified the use of CT screening in clinical practices and it is anticipated that GGO lesions will be detected more commonly [26].

GGO is defined as hazy opacity with increased lung attenuation that does not obscure underlying bronchial structures or pulmonary vessels at high-resolution CT [27] (Figure 1). Since GGO only refers to a morphologic finding on CT scans, nodules with GGO morphology may represent various pathologic entities such as inflammation, alveolar hemorrhage, eosinophilic lung disease, pulmonary lymphoproliferative disorder, organizing pneumonia, and neoplasm [28]. When GGO nodules are transiently observed and disappear thereafter, they are more likely to have been inflammation or hemorrhage. In contrast, GGO nodules that persist for more than several months could be a sign of early-stage ADC or its precursor [29-35]. Nakata et al. reported that GGO nodules persistently observed for several months turned out to be early-stage ADC such as BAC (AIS) or AAH [29]. The appeal of predicting the pathologic diagnoses based on noninvasive tests like CT scans led numerous attempts to elucidate the histopathologic findings of GGO nodules and correlate them with CT findings [29,31-33].

Prior to the introduction of such an 'attractive' high-resolution CT scanning, Noguchi et al. first conducted a large systematic study regarding the histologic features of small peripheral lung

ADC measuring 2 cm or less in diameter and then classified them into six subtypes [34]. Type A corresponds to localized BAC; type B, localized BAC with foci of collapsed alveolar structures; type C, localized BAC with foci of active fibroblastic proliferation; type D, poorly differentiated ADC; type E, tubular ADC; and type F, papillary ADC with evidence of compressive and destructive growth. Types A, B, and C ("replacement" early ADC), unlike types D, E, and F ("non-replacement" invasive ADC), represent a distinct group, because they show a lepidic growth pattern, which is characterized by tumor cell growth replacing normal alveolar lining cells. These tumors with replacement or lepidic growth pattern appear radiographically as a localized GGO lesion at high-resolution CT scans.

Since Noguchi et al. first proposed this novel classification, significant changes have been made in the World Health Organization (WHO) classification of lung ADC, which reflects our better understanding of the histopathologic features [36]. A major change made in the 1999 WHO classification and maintained in the 2004 WHO classification was the addition of AAH as a premalignant lesion [36]. AAH is defined as a localized proliferation of mildly to moderately atypical type II pneumocytes and/or Clara cells lining alveolar walls and sometimes, respiratory bronchioles when underlying interstitial inflammation and fibrosis are absent (Figure 2). The proliferation results in focal lesions of the peripheral lung, usually less than 5 mm in diameter [37]. On the other hand, BAC is a localized noninvasive ADC with a pure lepidic growth pattern restricted to neoplastic cells along preexisting alveolar structures and no evidence of stromal, pleural, or vascular invasion [36,38] (Figure 2). By definition, BAC corresponds to Noguchi type A and B lesions. Numerous clinical studies on solitary small (<2 or 3cm) lung ADC with a pure lepidic growth, that is, BAC have shown 100% disease-free survival after a curative-intent surgical resection [34,39-45].

However, if a GGO nodule (pathologically AAH or BAC) is not surgically resected and merely followed up by repeated CT scans without treatment, it increases in size and a solid component within the lesion tends to appear and increase in extent [46,47]. Takashima et al. conducted a serial CT follow-up study on the natural course of lung ADCs with GGO components and showed that GGO subsequently increased in size in 75% of patients, 17% of patients developed solid components within the nodule, and the solid portions increased in 23% patients [46]. Such a GGO nodule containing the solid component in it is called mixed GGO (part-solid nodule), whereas a GGO nodule without any solid component is called pure GGO (non-solid nodule) [32,48] (Figure 1). Solid components within GGO nodules pathologically represent areas of collapsed alveoli or fibroblastic proliferation and these mixed GGO nodules correspond to Noguchi type B and C lesions [34,46,49,50]. Greater the solid component corresponds with greater possibility of an invasive growth component [51-53]. Kodama et al. demonstrated that radiologic GGO components correlated with BAC components in pathologic specimens of ADC [54]. In the same context, AAH and BAC are manifested by pure GGO, whereas invasive ADCs include a greater solid component within GGO on CT scans. There have been several reports that lung cancer patients with smaller solid component had a much better prognosis than those with greater solid component [39,54,55]. For this reason, the diagnosis of BAC is based upon a premise that the entire tumor has no areas of invasion upon pathologic examination. It is therefore highly controversial to make a final diagnosis of BAC based on a small biopsy or cytology specimen

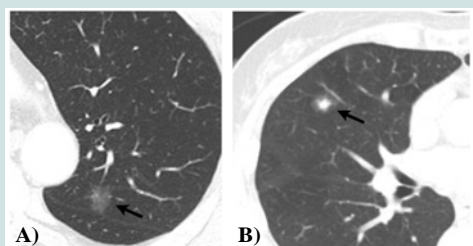


Figure 1: Images of ground-glass opacity (GGO) nodules on high-resolution computed tomography (HRCT) scans. GGO is defined as hazy opacity with increased lung attenuation that does not obscure underlying bronchial structures or pulmonary vessels at HRCT. When untreated, GGO nodules can gradually develop a solid component. Such a GGO nodule without any solid component is called pure GGO (non-solid nodule, A), whereas a GGO nodule containing the solid component in it is called mixed GGO (part-solid nodule, B).

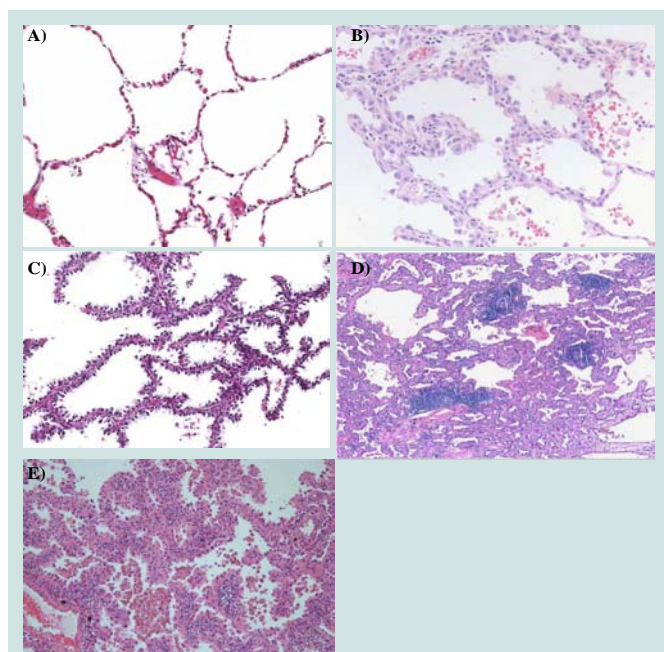


Figure 2: Photomicrograph (Hematoxylin-Eosin stain; original magnification, x 100) of normal lung structure (A), adenocarcinoma and its precursors (B to E). Atypical adenomatous hyperplasia (B) is a localized proliferation of mildly to moderately atypical type II pneumocytes and/or Clara cells lining alveolar walls, usually less than 5 mm in diameter. Adenocarcinoma *in situ* (C, formerly known as bronchioloalveolar carcinoma) is a localized noninvasive adenocarcinoma with a pure lepidic growth pattern restricted to neoplastic cells along preexisting alveolar structures and no evidence of stromal, pleural, or vascular invasion. Minimally invasive adenocarcinoma (D) has a predominant lepidic growth and small foci of invasion measuring 0.5 cm or less. When foci of invasion are greater than 0.5 cm, it should be classified into invasive adenocarcinoma with lepidic predominant (E).

alone. However, it should be noted that identification of small GGO nodules at CT in fact may lead to overdiagnosis and unnecessary treatment [56]. The question can be raised whether surgical resection is really needed for such a small pure GGO lesion, considering the fact that it grows very slowly or not at all during follow-up when untreated. In the same context, many surgeons demonstrated that limited surgical resection such as wedge resection rather than lobectomy is enough for small pure GGO lesions [57-59].

Nonetheless, many researchers and clinicians still use the term BAC for a broad spectrum of tumors from solitary small noninvasive lung tumors with a 100% 5-year survival [34] to invasive ADCs with minimal invasion that also have almost 100% 5-year survival [60,61] or mixed subtype invasive ADCs [62,63], creating great confusion in both clinical and research fields. Recently, new lung ADC classifications have been proposed by the European Respiratory Society, International Association for the Study of Lung Cancer, and American Thoracic Society [18]. The proposal strongly recommended discontinuing the use of the term BAC and instead recommended that the term 'AIS' should be used for small (≤ 3 cm), solitary ADCs with pure lepidic growth to define patients who should have 100% disease-specific survival upon complete resection. When small (≤ 3 cm), solitary ADCs have predominant lepidic growth and small foci of invasion measuring 0.5 cm or less, a new concept of 'minimally invasive adenocarcinoma (MIA)' is proposed to define patients who should have nearly 100% disease-specific survival upon complete resection. For invasive ADCs, comprehensive histological subtyping

should be used to assess histologic patterns semiquantitatively in 5% increments, choosing a single predominant pattern.

Concept of Multistep Progression

Based on clinical implications and pathologic findings, many researchers proposed a hypothesis of multistep carcinogenesis in which some lung ADCs arise from preneoplastic lesions called AAH, which progress to AIS, eventually developing into invasive ADC [15-18]. This concept was initially proposed in the field of colorectal cancer, in which colorectal carcinogenesis involves a multistep process from normal mucosa and inflammation, through early and late adenomas to invasive carcinoma [64,65]. If specimens for histologic analysis could easily be obtained repeatedly over time in the same patient, it would be an ideal method to elucidate the natural history of cancer. In colon cancer, this longitudinal study can be performed because it is relatively easy to obtain colon cancer specimens via an endoscopic approach. However, in lung cancer, such a procedure is more challenging especially in peripheral lung cancer, because it is rarely accessible via an endoscopic approach. Furthermore, it is much more difficult to obtain specimens if the lesion of interest is at an early stage such as AAH [66]. Although serial morphologic changes in imaging tests such as CT or positron emission tomography (PET) can be an alternative way to demonstrate the multistep progression of lung ADC [20,21], it cannot be guaranteed that radiographic features are equivalent to pathologic findings. Otherwise, it cannot help but compare individual lesions from different patients and a temporal assumption is inevitable in this cross-sectional method, which should be interpreted with caution. It should also be noted that the time point at which genetic or epigenetic alterations occur might differ from patient to patient, since it is still difficult to tell exactly when these alterations first occur. In addition, genes affected in a specific pathway might vary from tumor to tumor, because not all molecular changes will be fully penetrant and only a portion of tumors will gain a specific molecular change [67]. Furthermore, since lung ADC can no longer be considered a single type of tumor but rather a group of distinct subsets that arise from different molecular pathways, the concept of multistep progression carcinogenesis is not necessarily applicable to all ADCs [68].

In spite of all these limitations regarding the concept of multistep progression of lung ADC, there is sufficient pathologic and molecular evidence to substantiate this putative hypothesis of multistep carcinogenesis in lung ADC. First, AAH is frequently detected at the periphery of invasive ADC in lung cancer patients [17,19]. This phenomenon is relevant to the histopathological similarity between AAH and AIS [69-71]. Second, numerous molecular studies demonstrate that specific genetic alterations occur at similar frequencies in AAH and ADC, which reinforce the concept of AAH-AIS-ADC progression [66]. Multiple studies demonstrated close association between AAH and lung ADC including clonality [72,73], K-ras mutations [74,75], K-ras polymorphisms [76], epidermal growth factor receptor (EGFR) mutations [77], p53 expressions [78], loss of heterozygosities [79], methylations [80], telomerase overexpressions [81], eukaryotic initiation factor 4E expressions [82], epigenetic alterations in the Wnt pathway [83], and FHIT expressions [84]. For example, activating K-ras mutations are found with similar frequency in AAH and ADC [75,85] and the mutually exclusive features of EGFR and K-ras mutations observed in lung ADC are also found in AAH [74]. In addition, loss of heterozygosity events at specific chromosomal regions are detected at similar frequencies in

AAH and ADC [79,86]. Third, the postulated progression of AAH to AIS and then ADC is supported by conditional oncogenic mouse models in which EGFR or K-ras genes are activated. In both types of mice, AAH-like lesions are detected before ADCs develop, implying AAH as a precursor of ADC [87,88].

Genetic Alteration

Lung ADC arises from the accumulation of enormous genetic and epigenetic changes, giving advantages to neoplastic cells in cellular growth and/or survival with progression depending mainly on the balance between oncogene activation and tumor suppressor gene inactivation [89]. To date, over 100 oncogenes have been identified such as ras and tyrosine kinase receptors (EGFR, c-erb-B2 (HER-2/neu)). Many of these behave dominantly in that only one allele needs to be overexpressed to have effect [77]. In contrast, tumor suppressor genes behave as recessive genes and thus both alleles need to be inactivated either by epigenetic modifications (predominantly by promoter methylation), allelic deletion or mutation [71].

EGFR

Many AAH and AIS cases have been found to harbor EGFR gene mutation and these findings suggest that EGFR mutation has a critical role in the pathogenesis of lung ADC [75,77,90-93]. Furthermore, transgenic mouse models expressing mutated EGFR genes in type II pneumocytes develop AAH, BAC, and invasive ADC with a nonmucinous BAC component in the lung in a time-dependent manner [87,94]. Ligand binding to EGFR leads to receptor tyrosine kinase activation and a series of downstream signaling activation mediates increased cellular proliferation, migration, invasion and suppression of apoptosis [95]. Mutations responsible for its oncogenic activation and depending on the ligand (EGF, transforming growth factor- α , insulin-like growth factor-1, platelet-derived growth factor, amphiregulin) favor deregulated proliferation, differentiation, apoptosis and angiogenesis [71]. Somatic mutations of EGFR are characterized by two major hotspots, in-frame deletions in exon 19 and a specific missense mutation in exon 21 (ie, L858R), which constitute almost 90% of the EGFR mutations in lung ADC [90-95]. ADCs with these mutations have been reported in certain demographic populations, including female gender, never or light smoking status and East Asian ethnicity [101,102]. Histologically, they tend to be associated with ADC especially with nonmucinous BAC component [101,102]. More importantly, patients with lung ADC harboring these mutations are responsive to EGFR tyrosine kinase inhibitors such as gefitinib and erlotinib [96-101].

Based on these clinical circumstances, many investigators tried to elucidate the evidence of multistep progression from noninvasive to invasive tumors by comparing the incidence of EGFR mutation between AAH and AIS or MIA. The incidence of EGFR mutations in AAH varies from 3% to 44% [77,90,103]. Kozuki et al. reported that EGFR mutations were detected in 44% of AAHs (four of nine cases) [103], whereas Yoshida et al. showed that the incidence of EGFR mutation in AAH was only 3% (one out of 35 cases) [77]. The differences in these frequencies might be due to the sensitivity of the assays to detect EGFR mutations as there were fewer AAH cells than in the surrounding normal cells. Although the refinement of microdissection techniques coupled with improvements in PCR amplification has made it possible to study AAH lesions at the molecular genetic level [19], it remains technically challenging to conduct a molecular analysis for AAHs due to their small size. Nonetheless, the fact that EGFR mutations are detected even in AAH

is undeniable and this suggests that EGFR mutations occur early in the development of lung ADC before progression to invasive tumors (Figure 3).

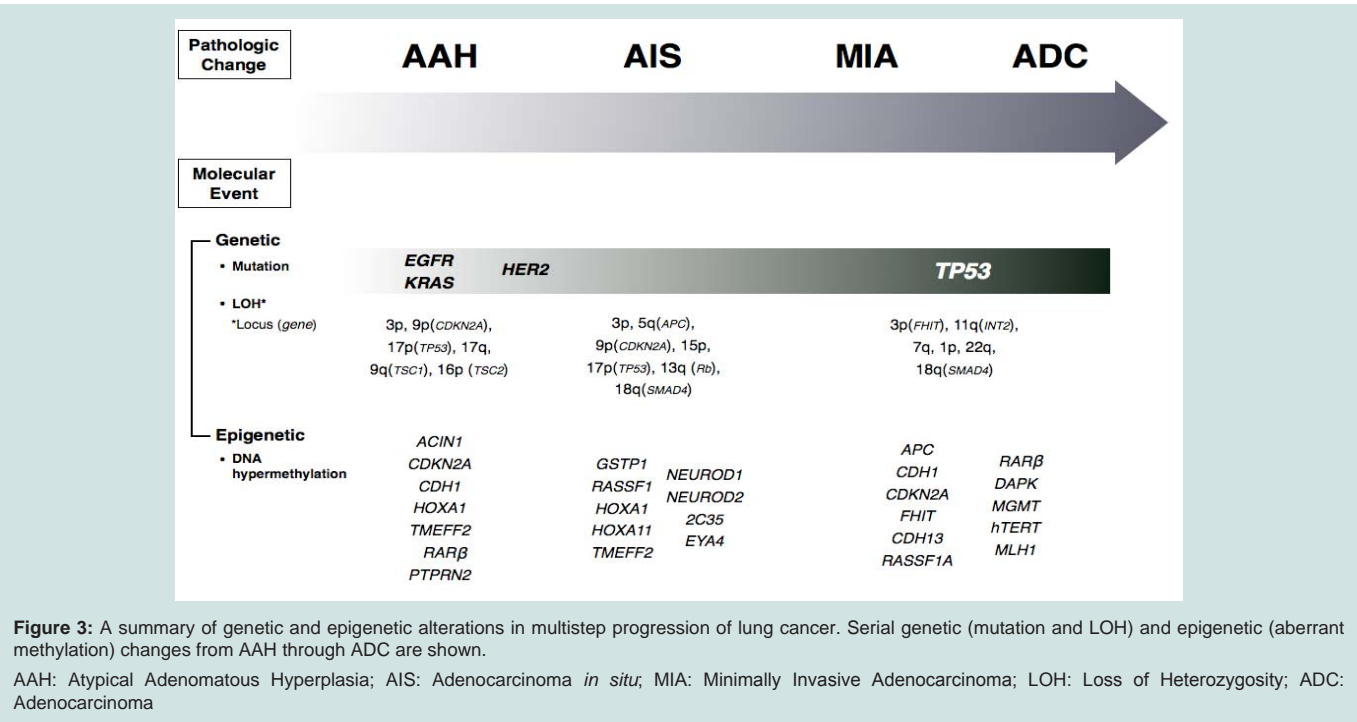
In contrast, the incidence of EGFR mutations in invasive ADC ranged from 23% to 50%, which seems to be relatively higher than that of AAHs [77,90,91,103]. Sakuma et al. reported that 15 of 17 (88%) pure nonmucinous BAC (AIS) and 49 of 65 (75%) invasive ADC with a nonmucinous BAC component had EGFR mutations [92]. These results imply that persistent EGFR signaling from activating EGFR mutations would be essential for the development and maintenance in lung ADCs with a nonmucinous BAC component [92]. Apart from these dissimilar frequencies of EGFR mutations based on the invasiveness of lung ADCs, the findings that EGFR mutations are detected in noninvasive tumors as well as invasive tumors indicate that EGFR mutation is an early event in the pathogenesis of lung ADCs [70,104-106].

While many researchers reported that EGFR mutation is an early event during the multistep progression of lung ADCs, EGFR amplification is considered to be a late event. Soh et al. found that EGFR mutations were present in 39.5% of noninvasive tumors and 50% of invasive tumors, while EGFR copy number was increased in 7.9% and 31.8% of noninvasive and invasive tumors, respectively [70]. They concluded that EGFR mutations occur early during the multistage pathogenesis of lung ADCs, but increased EGFR copy number is a late event during tumor development and plays a role in the progression of lung ADC independent of the initiating molecular events. This finding is in line with other reports that demonstrate EGFR mutations preceding amplification, with amplification favoring the mutant allele [107].

Like EGFR mutations, c-erb-B2 mutations are found most commonly in female Asian non-smokers with adenocarcinoma and are mutually exclusive with KRAS mutations [108]. HER2 protein expression was reported in 7% of AAH whereas up to 67% of lung ADC showed positive staining for HER2 protein [109,110]. HER2 overexpression in AAH and BAC suggests that the tyrosine kinase signaling pathway is altered by a variety of mechanisms before adenocarcinoma reaches its invasive stage. HER2 protein staining is also related to increased cellularity and pleomorphism of AAH. Therefore, it has been speculated that AAH is premalignant and abnormal c-erb-B2 proto-oncogene expression may occur in the later carcinogenic sequence [109].

K-ras

K-ras is a member of the ras family of oncogenes, and is located downstream of the EGFR surface receptor pathway. K-ras gene encodes a membrane-associated guanine nucleotide-binding protein of approximately 21 kD in size, designated p21-ras and activation of K-ras genes by mutations may contribute to malignant transformation [111-115]. K-ras mutations are found in 10 to 30% in NSCLC, especially ADCs, and over 90% of the mutations occurred in codon 12, occasionally in codon 13, and rarely in codon 61 [116-120]. Like EGFR mutation, K-ras mutation has also been detected in certain demographic populations, including non-East Asian male and ever smokers. Histologically, tumors tend to be associated with wild-type EGFR and contain mucinous BAC component features [102,117,118,121-123]. More importantly, K-ras mutations are found more frequently in patients who are resistant to therapy with EGFR tyrosine kinase inhibitors [124].



Initial studies mainly focused on the K-ras proto-oncogene as a likely target of mutational activation for several reasons [19]: (1) activating mutations of K-ras are detected in 24-50% of lung ADCs, but they are much less frequent in other subtypes of lung cancer [117,125]; (2) K-ras mutations are easy to detect because they involve straightforward base substitutions that predictably target codon 12 [117]; and (3) models of lung tumorigenesis addressing the chronological sequence of mutational events predict that K-ras activation is an early event that precedes malignant growth [85,126,127]. Therefore, K-ras mutations are the most extensively investigated genetic alterations in AAH and previous reports demonstrate that K-ras mutations are found in 15-50% of AAH [15,75,93,128,129]. These findings indicate that K-ras mutation is also an early event in the multistep carcinogenesis of lung ADC [15,75,93,128] (Figure 3).

Although K-ras mutation is frequently found in AAH, its incidence is relatively less frequent than that of EGFR mutations in AAH. K-ras mutations in AAH or in ADCs with a nonmucinous BAC feature were much less frequent than those with a mucinous BAC component [130]. Moreover, AAHs are strikingly similar to nonmucinous BAC, but quite different from mucinous BAC in histopathologic features. These observations suggest that AAH do progress sequentially to nonmucinous BAC, but not to mucinous BAC [130].

TP53

Mutation of the TP53 gene is one of the most frequent genetic alterations in lung cancer. TP53 is a tumor suppressor gene and its protein product is considered to play an important role in the control of cell cycle, DNA repair, apoptosis, and cell differentiation [131]. It reduces Rb phosphorylation and induces a G1-S checkpoint arrest to allow DNA repair or to drive cells to apoptosis mediated by Bax/Bcl-2 [71]. Its function is lost by mutation or inhibition of p53 pathway. Abnormalities of the TP53 gene, mainly missense mutations, result

in an impairment of the normal functions of the TP53 gene [131]. Since TP53 mutations lead to intranuclear accumulation of non-functional, stabilized p53 protein, immunohistochemical detection of p53 protein is an indirect measurement of TP53 gene mutations. Using this method, the expression of p53 protein in AAH, BAC and ADC has been studied by several researchers. Kerr et al. demonstrated that p53 protein was detected in 28% and 53% of AAH and ADC, respectively [109]. Kitamura et al. showed p53 expression in 5% to 8% of AAH lesions and 8% to 62% of BAC [78]. These findings indicate that the frequency of p53 nuclear accumulation sequentially increases from AAH to BAC and ADC. Therefore, this suggests that p53 overexpression might not be common even in the earliest stage of lung ADC and thus might be related to invasiveness in the tumor progression of lung ADC rather than initiation of tumor.

Loss of heterozygosity (LOH)

Inactivation of tumor suppressor genes is also an important factor in lung carcinogenesis. Loss of function of a specific gene occurs mainly by aberrant DNA methylation of its promoter region (will be described in the next section) and/or loss of heterozygosity (LOH) of the chromosomal region on which the gene is located. Many reports demonstrate that tumor and adjacent normal tissue from lung cancer patients contain LOH at distinct regions of chromosomes [132-136]. Deletions on chromosomal arms 3p, 2p, 12p, 9p, 8p, and 17p have been found to be widespread throughout the lungs of smokers even in the absence of overt histopathologic changes, suggesting that these alterations occur during the earliest stages of lung tumorigenesis [132-138].

LOH accumulates in crucial chromosomal regions in a stepwise manner during the multistep sequential progression of lung ADC [139]. When compared to histologically normal adjacent lung, AAH shows LOH of distinct regions of chromosomes 3p (18%), 9p (p16^{INK4a}, 13%), 9q (53%), 16p, 17q, and 17p (TP53, 6%), and these are changes also frequently detected in lung ADCs [140-143].

A detailed investigation of loci related to LOH in BACs and small ADCs, 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 [139]. This finding is consistent with the observed persistence of FHIT protein expression in AAH and BAC but loss of expression in invasive disease [84]. FHIT is a member of the histidine triad gene family involved in purine metabolism and it is known to work as a tumor suppressor gene. This late loss of FHIT is a very interesting finding in light of the observation of methylation of this locus in lavage fluid from cancer-negative cases [144], suggesting that genes become inactivated by different mechanisms at different times. Similarly, in the case of CDKN2A (p16^{INK4a}), biallelic inactivation through LOH combined with hypermethylation was seen in 22% of ADCs, providing a mechanism for progressive CDKN2A deregulation [145]. The p16 gene encodes cell cycle proteins (inhibitor of CDK4 and CDK6) and negatively regulates cyclin D-dependent phosphorylation of the Rb gene product, thereby inhibiting cell cycle progression from G1 to S-phase by sequestration of E2F [146,147]. These observations imply that deletions at chromosomal loci 5q, 9p, 11q, and 13q are relatively early events, which suggests that inactivation of the APC, p16 (CDKN2A), INT2, and Rb genes might be functionally associated with the pathogenesis of lung ADC and also indicate that deletions of 3p, 17p, 18q, and 22q increase significantly during the course of malignant progression (Figure 3).

Epigenetic Alteration

Silencing of various tumor suppressor genes by epigenetic alteration is also an important mechanism in human carcinogenesis [148]. Epigenetic changes in one allele and LOH or another epigenetic changes of the remaining allele can also result in biallelic inactivation of tumor suppressor genes [149-152]. Aberrant DNA methylation is a typical epigenetic change that has been extensively detected in nearly all types of cancer [153-158], including lung cancer [156-158]. DNA hypermethylation mainly occurs in the CpG islands located in the promoter regions of tumor suppressor genes, effectively silencing the gene without any accompanying alterations in the DNA sequence [153]. This phenomenon is very widely observed in lung ADC, which suggests that DNA hypermethylation plays a critical role in the pathogenesis of lung ADC.

Although DNA methylation is common in lung ADC, it is still unknown how frequently a specific gene is methylated at different steps during the multistep cancer progression. This is partly because AAH lesions provide little DNA due to their limited size. Nonetheless, many researchers have tried to determine the biological implication of DNA hypermethylation in the multistep tumor progression by comparing the frequencies at which loci are aberrantly methylated between noninvasive and invasive tumors. Licchesi et al. observed significant increase of hypermethylation of p16^{INK4a}, DAPK, MGMT, RAR, RASSF1A, and hTERT genes in the histologic progression from normal to AAH, with low grade or high grade atypia and finally adenocarcinoma [80]. Tanaka et al. reported that the aberrant methylation of p16^{INK4a} was significantly more frequent in invasive tumors (Noguchi type C) than in noninvasive tumors (Noguchi type A or B) [159]. Kubo et al. showed that the aberrant hypermethylation of p16^{INK4a}, RASSF1A and CDH13 was significantly more frequent in invasive tumors (Noguchi type C) than in noninvasive tumors (Noguchi type A or B) using a methylation-specific PCR assay and this finding suggests that methylation of these genes play roles in the

development of late-stage lung ADC, especially the acquirement of invasiveness [160].

Recently, Chung et al. showed that aberrant methylation of HOXA1, TMEFF2 and RARB occurred in noninvasive stages (AAH and AIS) and aberrant methylation of PENK, BCL2, RUNX3, DLEC1, MT1G, GRIN2B, CDH13, CCND2, and HOXA10 was more closely associated with the invasive stage than the noninvasive stage. These findings suggest that promoter CpG island hypermethylation occurs at an early stage of multistep pulmonary ADC (ADC) development and accumulates to the progression of ADC [161]. Selamat et al. performed a more comprehensive analysis for DNA methylation levels of histologically normal adjacent non-tumor lung, AAH, AIS, and invasive ADC at 15 CpG islands that are frequently affected in lung ADC using sensitive real-time PCR [162]. Loci in which DNA hypermethylation occurred in AAH (CDKN2A and PTPRN2) were different from those in AIS (2C35, EYA4, HOXA1, HOXA11, NEUROD1, NEUROD2 and TMEFF2) and invasive ADC (CDH13, CDX2, OPCML, RASSF1, SFRP1 and TWIST1) (Figure 3). This finding suggests that DNA hypermethylation of distinct loci develops at different time points during the development of lung ADC. Moreover, the fact that the number of methylated loci gradually increased from AAH to AIS and invasive ADC supports a model in which AAH and AIS are precursor lesions of a subset of lung ADCs.

Future Direction

Since the concept of multistep development and progression of lung ADC was postulated, supporting evidence has been accumulating in clinical, radiologic, pathologic and molecular studies. As mentioned earlier, however, a critical challenge that researchers are still facing in studying the molecular basis of this concept is limited availability of tissue samples. This is mainly due to the limited size of these early lesions like AAH, which are by definition smaller than 5mm and contain few cells from their alveolar structure [17]. Another reason is related to the fact that clinicians are reluctant to perform a surgery for early indolent nodules with GGO features. A possible solution to overcome this limitation is to find an alternative way of obtaining tissue samples by AAH or AIS-derived cell lines. This interesting approach was done by Shimada and coworkers, who compared a cell line derived from an AAH lesion (PL16T) with its normal counterpart (PL16B) [163]. Although there is still concern whether these cells maintain their AAH or AIS characteristics [66], this alternative method will potentially shed light on how to study the molecular alterations occurring during the development and progression from AAH to AIS and ADC. Another way to solve the limited specimen issue is to make better use of it by high-throughput technologies such as next generation sequencing (NGS) and tissue microarray. Data generated by high-throughput experiments need to be further analyzed by bioinformatics methods.

Apart from understanding the molecular basis of carcinogenesis, this effort to elucidate the concept of multistep progression can be used to develop a new biomarker specific for the different developmental stages of lung ADC. Moreover, considering that some epigenetic hits might be reversible in principle, newly-detected molecular alterations can be utilized to function as a potential therapeutic target or a great guide to chemoprevention.

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Acknowledgements

This work as supported by the Barbara Isackson Lung Cancer Research Fund (DJ), The Eileen D. Ludwig Endowed Fund for Thoracic Oncology Research (DJ), Uniting Against Lung Cancer (UALC) (IJK), and Mesothelioma Applied Research Foundation (MARF) (IJK).