

Original Article

Epidermal growth factor receptor gene amplification in atypical adenomatous hyperplasia of the lung

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Abstract: Atypical adenomatous hyperplasia (AAH) is postulated to be the earliest morphologic precursor lesion in lung carcinogenesis. The epidermal growth factor receptor (EGFR), one of the members of the Erb-2 family of receptors, is commonly expressed in non-small cell lung carcinoma (NSCLC). A subset of the patients with NSCLC has molecular abnormalities in the *EGFR* gene, including missense mutations and deletions and/or abnormal gene copy numbers, and the relative importance of each of these for patient outcome is an area of great interest. Recent reports show that *EGFR* mutations are rare or absent in AAH and are rare in bronchioloalveolar carcinoma (BAC). However, the *EGFR* gene copy number status in AAH is unknown. In this study, we examined the *EGFR* gene copy number status in lung adenocarcinomas, synchronous AAH, and BAC in surgical pathology resection specimens. *EGFR* gene copy number was analyzed by chromogenic in situ hybridization (CISH) using formalin fixed paraffin embedded tissue sections and EGFR probes as recommended by the manufacturer. A known positive case of high-grade glioma was used as a positive control. The results indicate that four of eight adenocarcinomas (50%) had more than five EGFR signals per nucleus, suggesting a gain in copy number. Interestingly, in four of nine cases of AAH (44.4%) more than three EGFR signals per nucleus were noted, with scattered cells showing up to 6 signals per nucleus. In addition, in five of 12 cases of BAC (42%), more than three EGFR signals per nucleus were noted. In the remaining cases two to three intranuclear dot-like peroxidase positive signals were present consistent with non-amplification of the gene. Our study reveals an abnormal *EGFR* gene copy gain in several cases of AAH. In our cohort, the rate of *EGFR* gene copy abnormalities in AAH appears similar to BAC and lower than in lung adenocarcinomas. These findings suggest that although *EGFR* gene copy abnormalities may be an early event in lung carcinogenesis, they are associated with tumor progression to invasive cancer and highlight the complexity of tumor morphogenesis.

Keywords: EGFR, lung cancer, chromogenic in situ hybridization, copy number

Introduction

Dysregulation of epidermal growth factor receptor (EGFR) signaling has been implicated in the pathogenesis of numerous carcinomas, most notably non-small-cell lung carcinoma (NSCLC) [1]. Recognized mechanisms of EGFR gain of function in NSCLC include somatic activating mutations in the exons encoding the tyrosine kinase domain [2] and *EGFR* gene amplification [3]. A hypothesis of multi-step tumorigenesis of lung adenocarcinoma starting with atypical adenomatous hyperplasia (AAH), progressing to bronchioalveolar carcinoma (BAC), and ultimately to invasive adenocarcinoma has been

proposed and widely considered [4-6]. Although there have been intensive efforts to characterize the molecular abnormalities of BAC and invasive adenocarcinoma, the molecular alterations of precursor AAH are an area of more recent investigation [7-11]. Furthermore, the genetic alterations in the EGFR signaling pathway, which have been shown to characterize the late-stage of certain lung adenocarcinomas, are not clearly defined in AAH [12]. Studies have identified somatic mutations of the *EGFR* gene in lung adenocarcinoma [13, 14]. However, *EGFR* mutations are relatively uncommon in AAH and likewise rare in BAC [8]. Similarly, copy number changes of *EGFR* have also been identified in

adenocarcinomas [15, 16] but to our knowledge, *EGFR* copy number changes in AAH have not been demonstrated in detail [17].

In this study, we therefore examined the extent of copy number variations in patients with AAH, BAC, and invasive lung adenocarcinoma by chromogenic in situ hybridization (CISH). CISH utilizes a peroxidase reaction to detect the locus of interest and can be performed in the clinical immunohistochemistry laboratory on formalin fixed paraffin embedded archival tissue and interpreted by standard light microscopy [15]. Recent studies from our group and others' have shown that CISH is a useful assay for detecting *EGFR* gene amplification in patients with NSCLC and has an important role for identification of patients with a high number of *EGFR* copies [15, 18, 19]. Furthermore, the reliability of CISH as a technique for detecting gene amplification has been established for HER-2 in breast cancer specimens [20-22]. We therefore used CISH to investigate whether early morphologically detectable lesions such as AAH manifest copy number changes in the *EGFR* locus. The specimens used in this study were from patients with lung adenocarcinomas and synchronous AAH, in addition to patients who exclusively had pure BAC.

Materials and methods

We studied ten consecutive patients who had NSCLC with synchronous AAH (seven of the ten) in their surgical pathology lung specimens, and 12 patients with BAC, who were treated at Brigham and Women's Hospital between 1999 and 2004. This study was approved by the local Institutional Review Board. Routine hematoxylin and eosin-stained slides from formalin-fixed, paraffin-embedded tissue sections were reviewed and classified according to the WHO criteria [5, 23] and staged according to the American Joint Committee on Cancer [24]. Representative areas containing carcinoma and AAH were selected for CISH analyses on 5 mm tissue sections.

Chromogenic in situ hybridization

Paraffin-embedded tissue sections were deparaffinized in two changes of xylene for 5 minutes each; xylene was removed in three washes of ethanol for three minutes each (100%, 100% and 95%) and the slides were washed in dis-

tilled running water for 5 minutes. The slides were placed in heated (>90°C) CISH Pretreatment Buffer (Invitrogen, Carlsbad, CA) and microwaved on high power for 30 minutes, then rinsed in distilled water for 5 minutes at room temperature. The tissue was digested for 10 minutes with pepsin digestion solution (Invitrogen, Carlsbad, CA) at room temperature, washed twice in distilled water for five minutes each, dehydrated in 90, 95, and 100% alcohol for 2 minutes each, then dried in a 37°C oven.

Five to seven µl of *EGFR* Amplification Probe (Invitrogen/Zymed, South San Francisco, CA) were applied to the designated area and a coverslip was applied and sealed with rubber cement. Slides were dried at 37°C, followed by probe denaturation at 95°C for five minutes and hybridization at 37°C overnight in a Thermobrite oven (MarketLab, Caledonia, MI). The slides were washed in 0.5% SSC for five minutes at room temperature, followed by 0.5% SSC for five minutes at 75°C, and water for five minutes at room temperature.

For immunodetection, slides were placed in 3% hydrogen peroxide in absolute methanol for ten minutes, and then washed in phosphate buffered saline with 0.025% Tween 20 (PBST) three times for 2 minutes each. Slides were incubated with nonspecific blocking solution (Zymed/Invitrogen, South San Francisco, CA) for 10 minutes at room temperature, then incubated with mouse anti-digoxigenin antibody for 30 minutes at room temperature, washed in PBST twice for two minutes each, and incubated with horseradish peroxidase-conjugated goat anti-mouse antibody for fifteen minutes, and finally washed again with PBST twice for two minutes each. Slides were then incubated with DAB Chromagen (Zymed/Invitrogen, South San Francisco, CA) for 30 minutes at room temperature, and washed in distilled water twice for two minutes each, and were counterstained with hematoxylin.

The CISH-prepared slides were examined at 400X magnification by bright field microscopy. Two pathologists (SS and LRC) examined the cases concomitantly and agreed on CISH scores; cases for which there was disagreement were further reviewed and a consensus was established. Both examiners were blinded to the FISH results. As in prior studies [15, 25], 200 tumor nuclei were examined in each case. In

EGFR copy number in AAH

Table 1. *EGFR* copy number in patients with atypical adenomatous hyperplasia (AAH), bronchioloalveolar carcinoma (BAC) and adenocarcinoma of the lung*

Case Nr	Age/Sex	Smoking		EGFR Copy Number	Surgical Procedure
		Pack-yrs	Type of Lesion		
1	50/F	40	AAH/ACA	3/3	RLL/RUL wedges
2	58/F	Unknown	AAH/ACA	2/2	RLL/RUL wedges
3	56/F	Never	AAH/ACA	5/7	RUL wedge
4	58/F	Unknown	AAH	2	Left lung, EPP
5	72/F	55	ACA	7	RUL wedge
6	62/F	45	AAH/ACA	4/10	LUL lobectomy
7	75/F	Never	AAH/ACA	2/2	RML lobectomy
8	69/F	12	AAH/ACA	6/2	LLL wedge
9	76/M	Unknown	AAH	2	RUL lobectomy
10	58/F	Unknown	AAH/ACA	6/9	LUL lobectomy
11	58/F	27	BAC	3	RUL wedge
12	59/F	Never	BAC	3	RLL wedge
13	49/F	Unknown	BAC	3	RUL lobectomy
14	66/F	25	BAC	5	RLL, wedge
15	69/F	Unknown	BAC	6	RUL wedge
16	80/M	10	BAC	3	RLL lobectomy
17	71/M	Occasion	BAC	6	RML wedge
18	66/F	Unknown	BAC	5	RUL lobectomy
19	56/M	20	BAC	5	RML wedge
20	75/F	Unknown	BAC	3	RLL wedge
21	79/M	40	BAC	3	LUL wedge
22	66/F	Unknown	BAC	2	LUL wedge

*ACA, adenocarcinoma; RLL, right lower lobe; RUL, right upper lobe; RML, right middle lobe; LLL, left lower lobe; LUL, left upper lobe.

cases where the number of signals per tumor nucleus varied between tumor cells, the range of signals counted was recorded for each case. Further validation of this work comparing CISH to FISH was recently published [15].

Statistical analysis

A geometric average was calculated from the range of CISH scores to represent the point estimate for each case. CISH scores were correlated between groups using nonparametric analysis (Spearman correlation).

Results

From the records of our institution, we identified seven patients with lung adenocarcinomas and synchronous AAH in the surgical pathology

specimens, one patient with adenocarcinoma alone, two patients with AAH alone, and 12 patients with BAC. The clinical and pathologic features of the patient groups are summarized in **Table 1**. All surgical pathology specimens were reviewed morphologically and classified according to the WHO criteria [23], and each was subjected to analysis of *EGFR* gene copy number using CISH. We sought to examine whether gene amplification of *EGFR* was present in precursor lesions of lung adenocarcinomas. Using surgically resected specimens of lung adenocarcinomas that demonstrated concurrent AAH, we used CISH to evaluate *EGFR* gene amplification. All specimens were from formalin fixed paraffin embedded tissue sections and *EGFR* probes were used as recommended by the manufacturer. Cases of BAC had features of involvement of alveolar walls by a proliferation of cuboidal

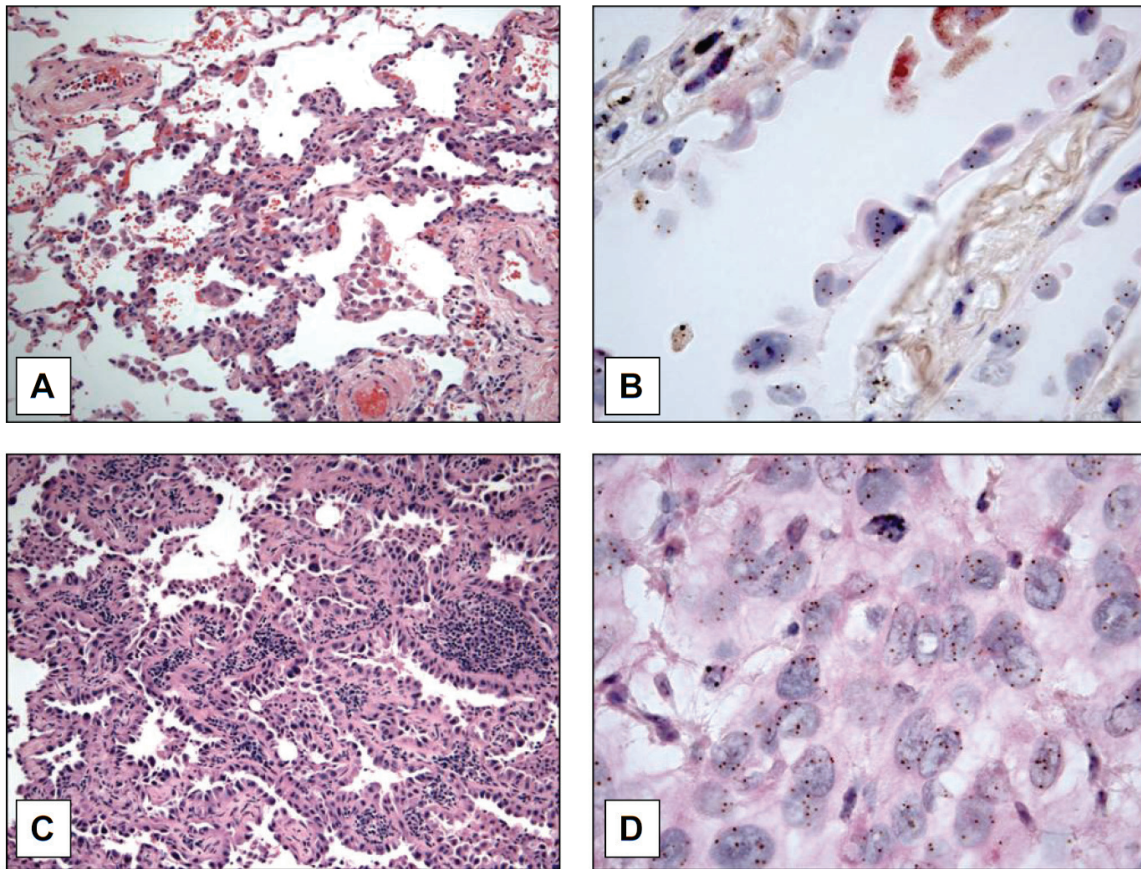


Figure 1. Surgical specimens from patients with atypical adenomatous hyperplasia (AAH), BAC (BAC) and adenocarcinoma of the lung. Panel A Atypical adenomatous hyperplasia (H&E 200x). Panel B Six EGFR signals per nucleus were noted in this case of AAH (H&E 1000x). Panel C Nonmucinous BAC. Uniform involvement of the alveolar walls by proliferation of cuboidal cells with severe atypia (H&E, 200x). Panel D Eleven EGFR signals per nucleus were noted in this case of adenocarcinoma (H&E 1000x).

cells with severe atypia, but without evidence of invasion. In contrast, the cases of AAH were localized proliferations of pneumocytes less than 0.5cm with marked hobnailing and mild to moderate atypia lining the alveolar walls and/or respiratory bronchioles. We were able to identify increased CISH signals representing amplification of the *EGFR* gene in cases of AAH (**Figure 1A, B**), suggesting the presence of increased *EGFR* gene copy number in this proposed earliest morphologic precursor of lung adenocarcinoma. We found up to six EGFR signals per nucleus (**Figure 1B**) in a case of AAH. In addition, we were able to detect increased copy numbers of *EGFR* by CISH in BAC. As previously reported, increased *EGFR* copy number was also identified in lung adenocarcinomas, with one example demonstrating up to ten EGFR signals per nucleus (**Figure 1D**).

To determine whether there might be a correlation between early lesions of lung adenocarcinoma with *EGFR* gene amplification, we examined whether there was a progressive increase in *EGFR* gene expression as a function of proposed tumor development. We found an abnormal *EGFR* gene copy gain in several cases of AAH (mean 3.5 signals, 95% CI 2.2 to 4.9) and a progressive increase in the rate of *EGFR* gene copy abnormalities to invasive ACA (mean 5.3 signals, 95% CI 2.4 to 8.1). In four of 9 cases of AAH (44.4%) more than three EGFR signals per nucleus were noted (**Figure 2**).

Discussion

In the current study, we used CISH to determine whether copy number changes to the *EGFR* locus might be an early event in lung tumorigene-

EGFR copy number in AAH

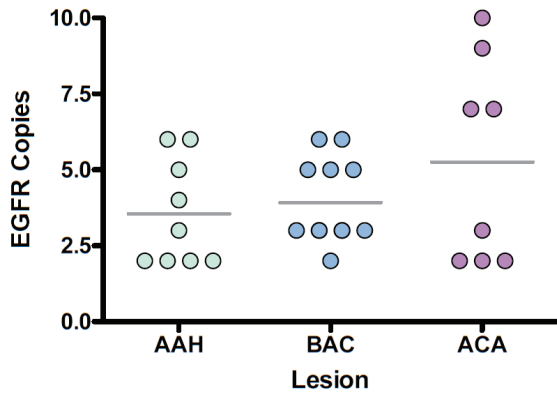


Figure 2. Scatter diagram illustrating the correlation between *EGFR* copy number and tumor progression, in AAH, BAC and lung adenocarcinoma. In four of 9 cases of AAH (44.4%) more than three *EGFR* signals per nucleus were noted. Horizontal bars represent the median value of *EGFR* copy number for each of the groups evaluated.

sis. We examined *EGFR* copy number changes in atypical adenomatous hyperplastic lesions, which are thought to represent the earliest morphologic entity that can be discriminated from either normal or reactive pulmonary epithelium. Although a previous report evaluated the prevalence of *EGFR* copy number abnormalities by CISH in AAH and lung adenocarcinoma [17], our findings are the first to demonstrate evidence for *EGFR* gene amplification in atypical adenomatous hyperplasia. The number of *EGFR* copies detected by CISH was higher in invasive adenocarcinoma than BAC and atypical adenomatous hyperplasia. Overall, we found that *EGFR* gene copy number in AAH is lower than in adenocarcinoma and very similar to that of BAC. Two other studies by Sakuma et al. found *EGFR* mutations by PCR-based methodology to be present in 32% of AAH cases, 88% of pure non-mucinous bronchioloalveolar carcinomas and 75% of invasive adenocarcinomas with a non-mucinous bronchioloalveolar component [26, 27]. Nevertheless further studies are needed to define this relationship. One limitation of our study was that most of the AAH were found concurrently in cases with invasive cancer. Ideally, examination of *EGFR* by CISH would occur in specimens devoid of an invasive lesion in order to definitively determine whether *EGFR* gene amplification is indeed an early event, or simply a correlate seen in cases of invasive adenocar-

cinoma with concurrent AAH. However, this would be difficult given that AAH lesions are small and are not usually or likely to be discovered by clinical or pathologic examination.

Activation of the *EGFR* signaling cascade has been shown to be due to a subset of mutations in the *EGFR* receptor kinase domain, which have been reported to occur with a frequency of 10% [1]. Such mutations appear to be more common in the adenocarcinoma histologic types and tend to be found in young, Asian women who have never smoked [28-30]. Notably, these patients appear to benefit substantially from inhibitors targeting *EGFR*. Other mechanisms of activation of the *EGFR* signaling cascade include gene amplification. Recent studies have highlighted the prognostic value of *EGFR* gene amplification in response to *EGFR*-inhibitor therapies [31-34]. Cappuzzo et al. showed that there was an increased responsiveness to kinase therapy in patients with lung adenocarcinomas that harbored increased *EGFR* gene copy number determined by FISH [31]. Similarly, Hirsch et al. demonstrated that increased *EGFR* gene copy number was associated with increased sensitivity to kinase inhibitors in patients with BAC's [34]. Adenocarcinomas with increased *EGFR* copy number were also found to correlate with *EGFR* overexpression in NSCLC and showed a trend towards poorer prognosis [16]. Importantly then, both mutations in *EGFR* and gene amplification are correlated with subsequent responsiveness to kinase inhibitor therapy. However, whether defects in kinase signaling are by themselves sufficient to induce malignancy is still unknown. Further research into the early mechanisms of lung tumorigenesis may reveal new strategies for the development of specific molecular therapeutics as well as provide for novel prognostic and diagnostic markers.

Our findings show that *EGFR* gene abnormalities are associated with tumor progression to invasive cancer and highlight the complexity of tumor morphogenesis.

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