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Immunohistochemical and Histopathological Study of Anaplastic Lymphoma Kinase and Tyrosine-kinase Receptor Expression in Bronchogenic Carcinoma

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Abstract

BACKGROUND: Adenocarcinoma of the lung is the most common tumor type of primary lung cancer and is characterized by heterogeneity on the molecular, clinical, and pathological levels. The presence of an anaplastic lymphoma kinase (ALK) fusion oncogene defines a molecular subset of non-small cell lung cancer with distinct clinical and pathologic features. Furthermore, the tyrosine-kinase receptor (C-kit) is considered to be expressed in various solid tumors, including carcinomas of the lung.

AIM: This study aims to correlate immunohistochemical (IHC) expression of ALK and C-kit with pathological features of lung carcinoma and to correlate IHC expression of ALK with IHC expression of C-kit in lung carcinoma.

MATERIALS AND METHODS: The material of this study consists of paraffin blocks of 60 cases of patients with bronchogenic carcinoma, IHC staining with ALK and C-kit then analysis of immunoreactivity scoring was done.

RESULTS: As regards ALK expression, 3 (5%) cases showed positive expression of ALK and 57 (95%) cases showed negative expression of ALK with no statistically significant correlation between the ALK expression and the histopathological type. While C-kit expression, 4 (6.7%) cases showed positive expression and 56 (93.3%) cases showed negative expression of C-kit with statistically significant correlation between the C-kit expression and the histopathological type.

CONCLUSION: There is an association between expression of c-kit and tumor histological type in lung carcinoma. Expression was notably significant among adenocarcinomas and small cell carcinomas.

Introduction

Adenocarcinoma of the lung is the most common histological type of primary lung cancer [1] and is a heterogenous tumor with diverse molecular, clinical, and pathological characteristics. Identification of molecular driver mutations and their therapeutic implications in lung adenocarcinoma have become an important area of research as evidenced by the abundance of genomic and mutational studies [2].

Rearrangements of the anaplastic lymphoma kinase (ALK) gene drive the malignant phenotype in 3–7% of primary lung adenocarcinomas [3]. The resulting fusion protein, most often a fusion with echinoderm microtubule-associated protein-like 4 (EML4), has a constitutively active tyrosine kinase domain. The small molecule drug crizotinib is a specific inhibitor of this kinase [4], and cases with the rearrangement respond to crizotinib treatment [5]. Therefore, accurate, rapid, and inexpensive identification of tumors growing under the influence of translocated ALK is needed [6].

Several studies have investigated the predictive value of pathological and morphological features in detecting ALK- rearranged tumors; however, the results of these studies have been inconsistent because of the limited number of ALK-rearranged tumors [3], [7]. Solid signet ring cell subtypes and cribriform pattern have been associated with ALK rearrangement in lung adenocarcinoma [7]. However, the comparative analysis of these histomorphological features and subtypes of ALK-rearranged lung adenocarcinoma based on driver oncogene mutations has not been clearly established in lung adenocarcinoma [8].

Small cell carcinoma can occur in any organ, but the great majority develops in the lung. Small cell lung carcinoma (SCLC) shows aggressive behavior and good prognosis. Some studies of SCLC have shown that kit protein is expressed in 30-100% of SCLC [9]. The difference of the percentage may be due to different primary antibodies used, staining methods, incubation periods, and interpretation of the immunostaining. In addition, one report showed that a few kit gene mutations were present in SCLC, but others did not [10].

Kit gene, mapped to 4q12, encodes tyrosine kinase receptor (C-kit) oncoprotein called kit (CD117). This molecule is transmembranous oncoprotein involved in tumorigenesis of some neoplasms including gastrointestinal stromal tumor, acute myeloid leukemia, mast cell neoplasms, germ cell tumors, melanoma, neuroendocrine carcinomas, large cell neuroendocrine carcinoma, and SCLC. The host spots of gene mutations are exons 9, 11, 13, and 17 of kit gene [11].

SCLC constitutes approximately 15% of all cases of primary lung cancer (Govindan *et al*.; 2006). SCLC is sensitive to chemotherapy and radiotherapy, but long-term survival is low and the majority of patients eventually develop progressive disease. There is a high rate of relapse even among patients who achieve a complete response. High levels of expression of C-kit and its ligand, stem cell factor, have been widely found in both SCLC tumors and established cell cultures [12].

Materials and Methods

Case selection

Ethical consideration

The protocol of the study was approved by the Ethical Committee of Faculty of Medicine, Cairo University (as the study performed on Archived blocks no informed consent needed).

The material of this study consists of paraffin blocks of sixty cases of patients with bronchogenic carcinoma, collected to private labs where the copies of their pathology reports were available at computer data base (From January 2016 to December 2017).

The specimens were obtained by either bronchoscopic biopsies, transbronchial lung biopsy, computed tomography guided core biopsy, tru-cut biopsy, or fiberoptic biopsy.

The data collected from the pathology request sheets enclosed with specimens included: Ages of all patients at diagnosis, sex, tumor subtypes, and grading.

Inclusion criteria

The following criteria were included in the study:
1. Cases of primary lung carcinomas with available clinic-pathological data
2. Specimens with adequate tumor tissue

Specimens with good histological orientation.

Exclusion criteria

The following criteria were excluded from the study:

1. Cases of primary lung carcinomas without available clinicopathological data
2. Cases with lost files or unavailable blocks.

Specimens with inadequate tumor tissue.

Steps of the work

Three sections (4 microns thick) were prepared from each paraffin block was re-cut by rotatory microtome, one of them was stained with hematoxylin and eosin for histopathological evaluation (for grading), and the other two were mounted on poly-L-Lysine-coated slides (Superfrost slides) and subjected to two immunohistochemical (IHC) markers: ALK and C-kit. All slides were examined under light microscope. Finally, IHC automated staining for each case was conducted for both ALK and C-kit, for evaluation as described in details late.

Immunohistochemical staining for ALK and C-kit

The sections were deparaffinized in xylene, then were hydrated through a series of graded alcohols (95–70%), distilled water, and phosphate buffered saline (at pH 7.5). The slides were then immersed in citrate buffer (pH 6) and were pretreated by microwave oven 800 w for 20 min for antigen retrieval. After a 25-min cooling period, the endogenous peroxidase activity was inhibited by incubation in 3% hydrogen peroxide (H_2O_2) for 5 min. After washing with Tris-buffered saline, the sections were incubated with the primary antibody for 1 h at room temperature. The primary antibodies are rabbit polyclonal antibodies (61-0028-2 Genemed) and (NBP1-85707, Novus Biologicals), diluted at 1:100 in primary antibody diluent (Genemed).

The sections were washed in Tris-buffer and incubated with avidin-biotin-peroxidase system for 30 min. Peroxidase reaction was detected by addition of diaminobenzidine tetrahydrochloride. All slides were rinsed well in tap water for 5 min then slightly counterstained with hematoxylin for 1–2 min and dehydrated in ascending alcohol. The slides were cleared in xylene for 3 changes, and then Canada balsam and cover slips were applied.

C-kit antibody labels breast epithelium, skin basal cells (melanocytes), spermatocytes, oocytes, and tissue mast cells. This antibody labeled 89% of seminomas (dysgerminomas), and 36% of small cell carcinomas of the lung [13].

The positive control for cases stained for ALK was normal small intestine (T cells), while the positive staining (normal) for C-kit was breast epithelium.

Evaluation of ALK and C-kit expression

All sections were screened to disclose the areas with well-preserved tissue architecture and cell morphology for scoring of immunoreactivities.

Areas with deterioration of tissue morphology due to processing were discarded in the analysis.

For the ALK stains, only cytoplasmic staining was considered as a positive reaction. The number of immunopositivity cells was semiquantitative estimated: No positive cells (−); <50% of the tumor cells staining positive (+); 50–75% of tumor cells staining positive (++); and >75% of tumor cells staining positive (+++). The staining intensity was graded on a scale from 0 to 3+ (0, negative; 1+, weak; 2+, moderate; 3+, intense) [14].

Staining of C-kit expression of tumors was scored as negative (0) if <5% of cells were positive, weak staining (+) if 5–25% of cells were positive, moderate (++) if 26–50% of cells were positive, and strong (+++) if >50% of cells were positive [15].

Statistical analysis

Computer software package Statistical Package for Social Sciences versus 21 was used in the analysis to estimate the correlation between ALK and C-kit immunoreactivity and clinic-pathological data for all cases (age, gender, histological type, and grade).

The significance of the results was assessed by determining the probability factor "p" value using Chi-square test. $p < 0.05$ was considered statistically significant.

Results

Description of the study variables

This retrospective study was conducted on 60 cases of bronchogenic carcinoma. Their ages ranged from 26 to 85 years with mean age 59.18 years.
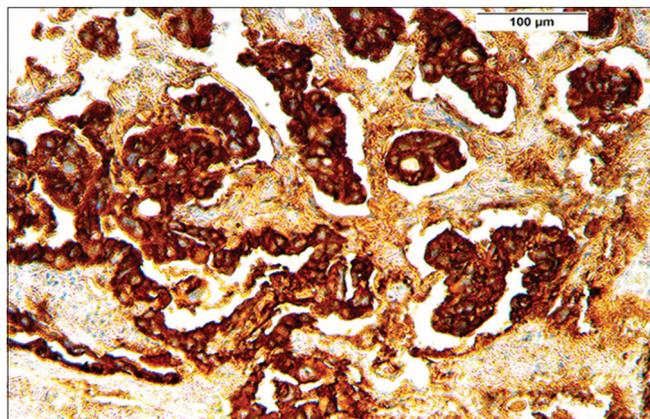

Figure 1: Moderately differentiated adenocarcinoma grade II, with strong cytoplasmic staining for anaplastic lymphoma kinase (score 3+) (200×)

As regards the histological type of lung cancer, 44 (73.3%) cases were classified as adenocarcinoma, 14 (23.3%) cases were classified as small cell carcinoma, and one case (1.7%) was adenosquamous

Table 1: The pathological data of the collected cases

Parameter	Number (%)
Sex	
Female	16 (26.7)
Male	44 (73.3)
Histological type	
Adenocarcinoma	44 (73.3)
Small cell carcinoma	14 (23.3)
Squamous cell carcinoma	1 (1.7)
Adenosquamous carcinoma	1 (1.7)
Grades of differentiation	
G2	28 (46.7)
G3	18 (30)
Undifferentiated	14 (23.3)

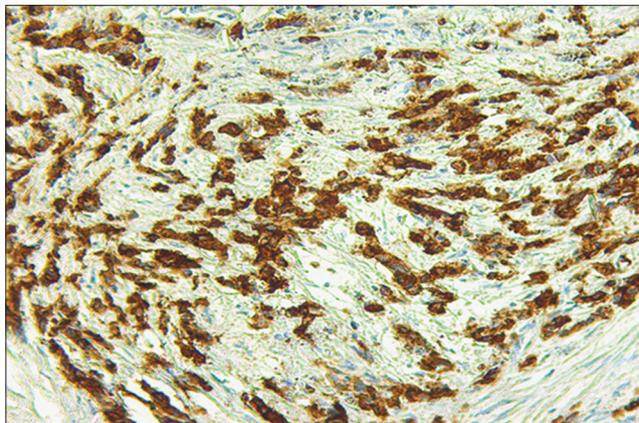carcinoma and other one case (1.7%) was squamous cell carcinoma.

Figure 2: Poorly differentiated adenocarcinoma grade III, with strong cytoplasmic staining for anaplastic lymphoma kinase (score 3+) (200×)

As regard sex distribution 44 (73.7%) were males and 16 (26.3%) were females. As regard sex distribution among lung adenocarcinomas, 28 (63.6%) were males and 16 (36.4%) were females.

As regards the histological grades, most of the case which constitute 28 cases (46.7%) were moderately differentiated, while the rest of the cases were distributed between poorly differentiated and undifferentiated constituting 18 (30%) and 14 (23.3%), respectively. The pathological data of the cases are summarized in Table 1.

As regards ALK expression, 3 (5%) cases showed positive expression of ALK and 57 (95%) cases showed negative expression of ALK (Figures 1-3).

Correlation between variables among the study group

Concerning ALK expression in non-SCLC (NSCLC), only 6.5% of cases showed positive expression of ALK.

Concerning ALK expression in lung adenocarcinomas, only 6.8% of lung adenocarcinomas showed positive expression of ALK. Correlation between the ALK expression and histopathologic type among the study group showed a statistically non-significant correlation (p = 0.76).

Correlation between the tumor histological grade and expression of ALK among the study group
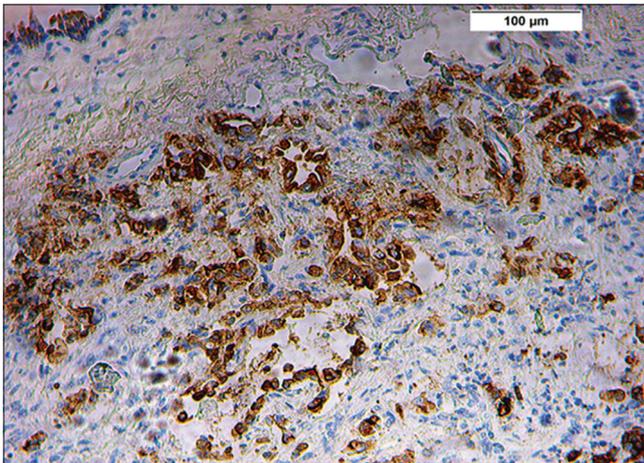
Figure 3: Poorly differentiated adenocarcinoma grade III showed moderate cytoplasmic staining for anaplastic lymphoma kinase (score 2+) (200×)

showed three positive cases and distributed as two cases were moderately differentiated, one case was poorly differentiated, no well differentiated, or undifferentiated cases. The correlation was statistically not significant (p = 0.601).

As regard ALK scoring, two of the positive cases showed cell positivity >75% (score 3) and the other positive one showed positive cells ranged between 50% and 75% (score 2). Their correlation with NSCLC and SCLC was statistically not significant (p = 0.618).

Similarly, the correlation between ALK scoring and the different histologic types was statistically not significant (p = 0.979).

As regards C-kit expression, 4 (6.7%) cases showed positive expression and 56 (93.3%) cases showed negative expression of C-kit (Figures 4).
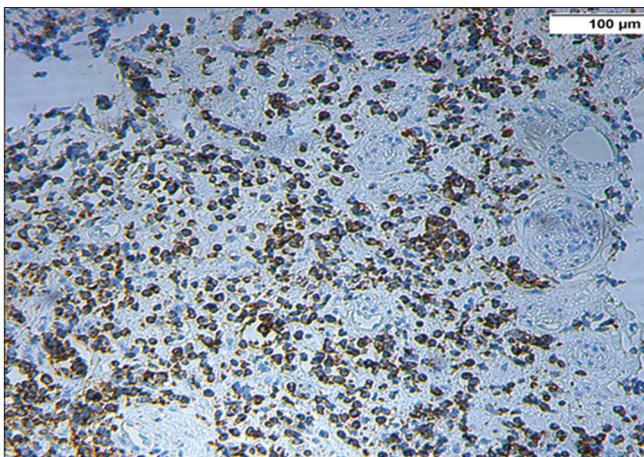

Figure 4: Small cell carcinoma, undifferentiated showed strong cytoplasmic staining for tyrosine-kinase receptor (200×)

Expression of C-kit was statistically significant among small cell carcinoma. Both cases of adenocarcinoma and squamous cell carcinoma were negative for C-kit. Correlation between the tumor histological type and expression of C-kit among the study group showed a statistically significant correlation (p < 0.003).

Correlation between the tumor histological grade and expression of C-kit among the study group showed that the four positive cases for C-kit were small cell carcinoma which was by their name considered as undifferentiated carcinoma and no well, moderate. or poor differentiated cases. The correlation was statistically significant (p = 0.001). All data regarding the correlation parameters of ALK and C-kit were summarized in Table 2.

Table 2: Correlation between expression of ALK and C-kit clinicopathological

Histopathological type and grade	ALK +ve (%)	ALK –ve (%)	p-value	C-kit +ve (%)	C-kit –ve (%)	p-value
Histological type						
Small cell carcinoma	0 (0)	14 (100)	<0.76	4 (28.6)	10 (71.4)	<0.003
Adenocarcinoma	3 (6.8)	41 (93.2)		0 (0)	44 (100)	
Others	0 (0)	2 (100)		0 (0)	2 (100)	
Histological grade						
G2	2 (7.1)	26 (92.2)	0.601	0 (0)	28 (100)	0.001
G3	1 (5.6)	17 (94.4)		0 (0)	18 (100)	
Undifferentiated	0 (0)	14 (100)		4 (28.6)	10 (71.4)	

ALK: Anaplastic lymphoma kinase; C-kit: Tyrosine-kinase receptor.

As regard C-kit scoring, two of the four positive cases showed cell positivity >50% (score 3). The other two were equally distributed between score 1 and 2. The correlation was statistically not significant (p = 0.119).

Discussion

Adenocarcinoma of the lung is the most common histological type of primary lung cancer [1] and is a heterogenous tumor with diverse molecular, clinical, and pathological characteristics. Identification of molecular driver mutations and their therapeutic applications in lung adenocarcinoma has become an important area of research as evidenced by the abundance of genomic and mutational studies [2].

Although the EML4-ALK fusion gene is minor genetic abnormality in NSCLC [7], the incidence of lung cancer is increasing in many countries, the absolute number of lung cancer patients harboring the EML4-ALK fusion gene is not trivial [16].

The present study demonstrated that cytoplasmic expression of ALK was significantly among lung adenocarcinomas. All cases of small cell carcinoma were negative for ALK. However, no significant correlations were regarded with tumor grade.

In our study, as regard sex distribution among lung adenocarcinomas, 28 (63.6%) were males and 16 (36.4%) were females. Indeed, in one study addressing lung adenocarcinomas, an imbalanced sex ratio was detected, with women exhibiting a 2.2-fold relative risk for an alteration [17], while in another study, there was a nearly balanced sex ratio [18].

In our study, as regards the histopathological type of bronchogenic carcinomas, 73.3% of cases

were classified as adenocarcinomas. Similar finding was obtained by Martinez *et al*., 2013 and found that three-fourth of the cases were classified as adenocarcinoma [18].

In the present study, we described an association between expression of ALK and lung adenocarcinomas. 3 out of 44 cases of lung adenocarcinomas were positive for ALK, while 4 out of 14 cases of small cell carcinoma showed C-kit expression.

Concerning ALK expression in NSCLC, in our study, only 6.5% of cases showed positive expression of ALK. ALK protein was expressed in 10.7% of 262 NSCLC tumors [19]. Paik *et al*., 2011 reported an association between expression of ALK and non-small cell carcinomas in 8.6% of 465 cases [20]. 5.1% of 99 cases were ALK positive [18]. IHC-positive results were demonstrated in 4.6% of 3244 cases [17]. ALK protein expression was detected in 4.2% of 473 cases [16]. ALK was detectable by IHC in 4% of 46 NSCLC specimens [21], 3.4% of 523 cases were ALK (+) by IHC [22], 2.9% of 377 cases were positive by IHC [23]. Selinger *et al*., 2013, identified an ALK gene rearrangement in 1% of 594 cases, although the intensity of staining was weak in some cases [24]. IHC is a reliable screening tool for identification of ALK rearrangement in NSCLC and is antibody dependent.

Concerning ALK expression in lung adenocarcinomas, in our study, only 6.8% of lung adenocarcinomas showed positive expression of ALK. Similar finding was obtained by To *et al*., 2013, who found ALK expression in 5.9% of cases [25]. A slightly higher percentage was also obtained by Zhou *et al*., 2014 in a large series of cases of lung adenocarcinomas that showed a significant correlation with the ALK expression (8.4%) [26]. Different findings were reported by Boland *et al*., 2009 [27], Takeuchi *et al*., 2009 [28], Jokoji *et al*., 2010 [29], Sakai *et al*., 2013 [30], Ying *et al*., 2013 [31], Han *et al*., 2013 [32], Zhou *et al*., 2014 [26], and Wang *et al*., 2014 [33]. IHC might provide a reliable and cost-effective diagnostic approach in routine pathologic laboratories for the identification of suitable candidates for ALK-targeted therapy.

Sample selection (histological type and tumor grade), number of cases analyzed, and differences in staining evaluation may be held responsible for the observed discrepancies between different studies.

Concerning scoring of ALK expression in NSCLC, in our study, only two cases (4.3%) of IHC NSCLCs showed an intense staining (3+) while the remaining one case (2.2%) showed a moderate staining (2+), the present work supports a previous study by Paik *et al*., 2011 [20], in which ALK protein expression was consisting of IHC scores of 1+ (3%), 2+ (2.1%), and 3+ (3.4%). Similar results were observed by Park *et al*., 2012 [19], who had observed that NSCLC patients with IHC score of 3 (3.4%), score of 2 (4.2%), and score of 1 (1.9%). Moreover, in another

study on lung cancer, cases of NSCLC were assessed as 1+ (faint cytoplasmic staining, 21%), 2+ (moderate, smooth cytoplasmic staining; 3%), or 3+ (intense, granular cytoplasmic staining in ≥10% of tumor cells; 8%) [14]. Based on these findings, the IHC assay reliably detected NSCLC with ALK and may be useful as a screening method to identify these tumors.

Results of present study as regards correlation between scoring expression of ALK and lung adenocarcinomas support a previous study by Zhou *et al*. 2014, who found an association between scoring expression of ALK and lung adenocarcinomas [26]. Other study has observed a correlation between scoring expression of ALK and lung adenocarcinomas [34].

One report on lung adenocarcinomas showed 5.9% of ALK-positive tumor cells demonstrated strong and diffused granular staining in the cytoplasm [25]. In another studies, they also found similar results for cytoplasmic staining (7%) [26], which was similar to our results (6.8%). However, other studies on lung adenocarcinomas had found similar findings (6.1%) [34]. These results suggest a possible role for IHC screening to be the first step in ALK testing algorithms, which can maximize the detection percentage of ALK positive case.

Small cell carcinoma can occur in any organ, but the great majority develops in the lung. SCLC shows aggressive behavior and poor prognosis [9]. SCLC constitutes approximately 15% of all cases of primary lung cancer [35].

Studies have confirmed that protein over expression or mutations of KIT are involved in growth and development of a variety of cancers. However, little is known about data of gene mutation and protein expression in SCLC patients [36].

The present study demonstrated that expression of C-kit was significantly associated with tumor histological type. Expression was notably significant among small cell carcinoma. All cases of non-small cell carcinoma were negative for C-kit. However, no significant correlations were regarded with tumor grade.

Many studies have reported that C-kit expression was related to SCLC [37], [38], [39], [40], [41], [42], [43]. KIT protein is expressed in a high percentage of SCLC tumors.

One study on lung cancer [40] failed to find a correlation between expression of C-kit and non-small cell cancer, which was similar to our study. Results of C-kit expressions were not so effective in whole histologically grouped patients.

In our study, concerning gender in lung small cell carcinoma, male gender was exclusive (100%). Several authors suggested a possible role for predilection of male gender in small cell carcinoma (56.6%) [37].

Our study stated that there is an scoring of expression of C-kit in 28.4% of SCLC; 14.2% showed

strong staining, 7.1% were moderate positive, and 7.1% were weak positive, whereas (71.4%) were negative. Burger *et al.*, 2003 stated that the expression of C-kit, estimated by IHC, was demonstrated in 64% of SCLC samples; 41% showed moderate to strong staining and 23% were weakly positive, whereas (36%) were negative [38]. The expression of KIT had no significant impact on survival.

Sample selection (histological type and tumor grade), number of cases analyzed and differences in staining evaluation, genetic and geographic variations may individually or in combination be held responsible for the observed discrepancies between different studies.

Recommendation of the Study

Although, it was recommended that IHC testing is clearly at least useful in routine practice as a screening test. The danger of missing treatable cases using this method (i.e., fluorescence *in situ* hybridization-positive, IHC-negative, and crizotinib-sensitive tumors), especially when specimens contain adequate material. Thus, further investigations such as re-biopsy and repeated IHC may be helpful. The accurate and timely identification of patients with ALK-rearranged lung adenocarcinomas is likely to be of therapeutic importance. We believe that IHC is a preferred method for identifying ALK-rearranged lung adenocarcinomas in routine clinical practice.

Ethical Approval

The protocol of the study was approved by the Ethical Committee of Faculty of Medicine – Cairo University.

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