© Kamla-Raj 2018 Int J Hum Genet, 18(1): 7-12 (2018)
DOI: 10.31901/24566330.2018/18.1.673

Frequency of Anaplastic Lymphoma Kinase (ALK) Rearrangement in Turkish Patients with Non-small Cell Lung Carcinoma

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KEYWORDS Cancer. Gene. Lung. Mutation. Profiling

ABSTRACT This paper aimed to document anaplastic lymphoma kinase (*ALK*) rearrangements in non-small cell lung carcinoma (NSCLC) patients retrospectively and to determine the frequency of this mutation in a population of Turkish patients. Samples of 503 patients referred to a regional reference laboratory with NSCLC diagnosis were included. Fluorescence in situ hybridisation (FISH) with a multicolour break-apart *ALK* probe was used to screen for *ALK* gene rearrangements. Overall, 45 (9%) of the 503 tumour samples were positive for *ALK* rearrangements. The frequency of *ALK* positivity was 9.89 percent (38/384) in male patients and 5.88 percent (7/119) in female patients (p=0.361). The frequency of *ALK* gene rearrangement detected by FISH was nine percent. This study is first to demonstrate the frequency of *ALK* rearrangements in NSCLC patients in Turkey. Given that *ALK* rearrangements provide a target for NSCLC therapy, molecular profiling should be performed for all NSCLC patients.

INTRODUCTION

Lung cancer, which is histologically classified by the World Health Organization (WHO) as non-small cell lung carcinoma (NSCLC, 85%) and small-cell lung carcinoma (SCLC, 15%) (Dai et al. 2012), is one of the most commonly diagnosed malignancy and a leading cause of cancer-related deaths both worldwide (Zheng et al. 2017) and in Turkey (Public Health Institution 2009). According to the estimates of American Cancer Society, in 2018, there will be approximately 234,030 new cases of lung cancer in the USA and approximately 154,050 deaths from lung cancer (American Cancer Society 2018). Depending on the medical status and disease stage of patients, general treatment strategies for lung cancer include surgery, radiation therapy, chemotherapy, and targeted therapy. Moreover, companion diagnostic testing of biological markers is now an essential part of personalised cancer treatments (Shojaee et al. 2017).

Until the last decade, systemic treatment of metastatic NSCLC has been limited to platinum-based doublet chemotherapy that yields a response rate of approximately twenty percent and provides a median survival of 8 months (Absenger et al. 2017). In the treatment of NSCLC, targeted kinase inhibitors can be used in the presence of specific genetic alterations, particularly with a high efficacy in tumours having kinase-associated molecular alterations (Chan and Hughes 2015). Accordingly, identification of the sensitising mutations of the epidermal growth factor receptor (*EGFR*) gene and the anaplastic lymphoma kinase (*ALK*) rearrangements (such as echinoderm microtubule-associated protein-like 4 [*EML4*]-*ALK*) have led to the use of *EGFR* tyrosine kinase inhibitors (erlotinib, gefitinib, afatinib) and *ALK* inhibitors (crizotinib and, ceritinib), respectively, for the treatment of NSCLC. These discoveries have provided selected lung cancer patients with targeted, personalised treatment options and thereby with clinically significant benefits (Langhammer and Scheerer 2017). Thus, the use of molecular testing of NSCLC is now highly recommended also for metastatic patients (Gao et al. 2017). Moreover, the Nation-

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al Comprehensive Cancer Network (NCCN) guidelines for the treatment of NSCLC include *ALK* testing and recommend treating *ALK* positive patients with kinase inhibitors even if the testing is performed after planned treatment initiation (NCCN 2017).

Recent studies have reported *ALK* gene rearrangements to be present in three to five percent of NSCLC patients (Gao et al. 2017; Lin et al. 2017). This rate was reported relatively within a broader range (3-13%) in the earlier studies (Dai et al. 2012; Kwak et al. 2010; Takanashi et al. 2015; Martelli et al. 2009). *ALK* gene rearrangements in NSCLS patients have been reported mostly in those with adenocarcinomas and in younger patients with minimal or no smoking history (Dai et al. 2012; Kwak et al. 2010). It has been defined almost impossible that *EGFR* mutations and *EML4-ALK* rearrangements coexist in the same tumour (Lo Russo et al. 2017); that is, *EGFR* and *KRAS* mutations are not typically observed in tumours with *ALK* gene rearrangement. Nevertheless, many recent observations have appeared to indicate that this is not true in all cases (Lo Russo et al. 2017). However, while patients with lung cancer harbouring *ALK* rearrangements do not respond to *EGFR*-specific tyrosine kinase inhibitors, they can benefit from treatment with *ALK* kinase inhibitors (Dai et al. 2012). Besides lung cancer, *ALK* gene rearrangement also has a role in other pathologies such as radiation-induced adult-onset papillary thyroid carcinoma (Hamatani et al. 2012) and neuroblastoma (Parodi et al. 2011).

Objectives

The aim of the present paper was to retrospectively document the patterns of *ALK* rearrangements in NSCLC patients and examine the frequency of this mutation in a Turkish patient population.

MATERIAL AND METHODS

The paper included 503 samples of patients who were referred to a regional reference laboratory for molecular profiling with a diagnosis of NSCLC. The patients had no kinship; all were unrelated, newly diagnosed, and suffering from the disease (with or without metastasis). All patients were histopathologically diagnosed as adenocarcinoma (large cell or NSCLC) – not otherwise specified. The median patient age was 61 years (range, 24-88 years) and 76.3 percent of the patients were male. All patients provided written informed consent to allow their samples to be analysed.

In this paper, a US Food and Drug Administration (FDA)-approved method (Dai et al. 2012), involving fluorescence in situ hybridisation (FISH) with a multicolour break-apart *ALK* probe was used to detect *ALK* locus rearrangements. For this purpose, the specimens were transferred to the genetics laboratory in paraffin-embedded tissue blocks. The blocks were sliced into sections that were several microns in thickness and the slices were then mounted on poly-lysine coated slides. Deparaffinization was carried out with a ThermoBrite Elite device (Leica Biosystems Inc, IL, USA) and using Clearene solvent instead of xylene. Other steps included washing with hundred percent, eighty-five percent, and seventy percent ethanol in series and then with 2× saline-sodium citrate (SSC) buffer with 0.2 M HCl and incubation using commercially available pre-treatment solution and then using pepsin solution.

Denaturation and hybridisation of the tissue samples on the slides were performed using the ThermoBrite system (Abbott Molecular Inc, IL, USA). After a 5 minute of an incubation period in 2× SSC buffer, dehydration was performed with ethanol in series (70%, 85%, 100%). After the slides were air dried, the probe was added and the coverslips were sealed with rubber cement to prevent evaporation of the probe solution. Denaturation was performed at 80°C for 5 minutes and overnight incubation was performed at 37°C using the ThermoBrite system.

Two types of commercially available probes were used to detect *ALK* rearrangements: 1) Vysis LSI ALK Dual Color, Break Apart Rearrangement Probe (Abbott Molecular, Abbott Park, IL, USA) consisting of two fluorescently labelled probes that flank the *ALK* gene breakpoint and produce one red and one green signal and, 2) the Kreatech KBI-10747 (Leica Biosystems Inc, IL, USA) for *ALK* 2q23 break.

Fluorescence signals were evaluated using an epifluorescence microscope (Olympus Life Science Solutions, Global Inc, USA) and the images were acquired with a CCD camera. FISH for *ALK* rearrangements was considered positive in the presence of the classic break-apart pattern or the atypical pattern (Yi et al. 2011). In the classic break-apart pattern, one fusion signal and two separated orange and green signals (a distance of $\geq$2 signal diameters between the two signals) is observed, whereas in the atypical pat-

tern, a single red signal without a corresponding green signal are observed in addition to the fused signals (Yi et al. 2011). The sample was considered positive if the rearrangement was observed in > fifteen percent of at least 60 counted cells in at least one tissue core sample (Zito Marino et al. 2015).

Data analysis was performed using the chi-square test for independence and comparisons of the groups.

RESULTS

In this paper, *ALK* FISH test was performed on 503 samples from NSCLC patients.

Overall, 45 (9%) of the 503 tumour samples had altered *ALK* signals that were consistent with "*ALK* gene rearrangement" and were considered "positive" (Table 1). Intact *ALK* genes revealed a two fusion signal pattern (Fig. 1). In all positive cases, the pattern of one split orange and green signal was indicative of an *ALK* gene rearrangement and one single fusion signal from the intact *ALK* gene was also observed (Fig. 2). Among the different variant signals, the most common was *ALK* gene deletion observed in 11 cases (Fig.3). The signals indicating gains of intact *ALK* signals that were consistent with polysomy were observed in 24 cases (Fig. 4).

Table 1: Distribution of tumour samples according to the *ALK* gene rearrangement

ALK gene rearrangement	*n (%)*
Negative	422 (83.9)
Positive	45 (8.9)
Other	36 (7.2)
Total	503 (100.0)

ALK: Anaplastic Lymphoma Kinase

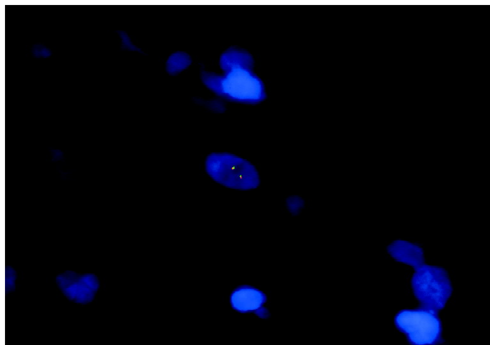

Fig. 1. Normal *ALK* signal pattern. Note the two fusion signals.

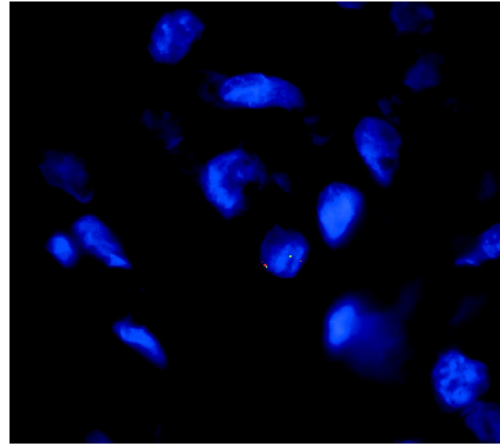

Fig. 2. Typical *ALK* rearrangement signal. One of the fusion signals are split due to the rearrangement

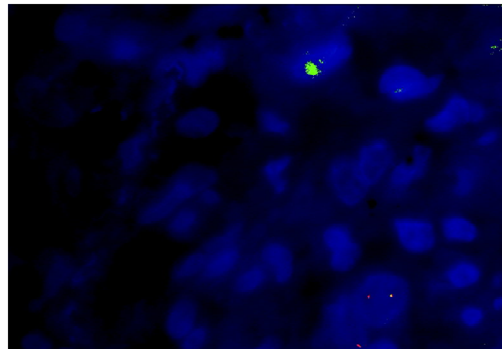

Fig. 3. Atypical signal pattern due to a partial deletion of the gene. 5' signal of the fusion is lost leaving an intact single red signal of the distal part.

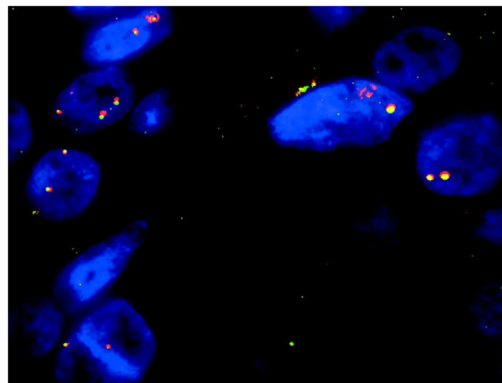

Fig. 4. Two different cells comprising of more than two fusion signal patterns that indicate amplification of the gene.

Of the *ALK*-positive cases, 38 were males with a median age of 57 years (range 24-84 years) and 7 were females with a median age of 53 years (range, 36-73 years) (Table 2). The frequency of *ALK* positivity was 9.89 percent (38/384) in male patients and 5.88 percent (7/119) in female patients. No significant difference was determined between male and female patients regarding the frequency of *ALK* positivity (χ^2=2.035, p=0.361).

Table 2: Gender distribution of cases according to the *ALK* gene rearrangement

ALK gene rearrangement	*Malen (%)*	*Female n (%)*	*Total*
Negative	320 (75.8)	102 (24.2)	422 (100.0)
Positive	38 (84.4)	7 (15.6)	45 (100.0)
Other	26 (72.2)	10 (27.8)	36 (100.0)
Total	384 (76.3)	119 (23.7)	503 (100.0)

ALK: Anaplastic Lymphoma Kinase

The median age of *ALK*-positive cases (n=45) was 57 years (range, 30 to 84 years; Table 3). There was a significant difference among the three groups (*ALK*-positive cases, *ALK*-negative cases and the others) with respect to age distribution (p=0.046); thus, no specific relation was found between the age and *ALK* rearrangement. No specific common clinical feature was worth to note among *ALK*-negative patients.

Table 3: Age distribution of cases according to the *ALK* gene rearrangement

ALK gene rearrangement	*Age, year*		
	Mean± SD	*Median*	*Minimum-Maximum*
Negative	61.3±10.8	61.0	27.0-88.0
Positive	56.8±13.2	57.0	30.0-84.0
Other	58.8±12.3	59.5	24.0-78.0

DISCUSSION

This paper is the first to demonstrate the frequency and patterns of *ALK* rearrangements in clinical specimens from a large cohort of Turkish patients with NSCLC who were referred for molecular profiling. FISH analysis of *ALK* gene rearrangements revealed a frequency of nine percent of *ALK* positivity in the present cohort. This finding was consistent with those reported in earlier studies indicating the rate of *ALK* positivity to be between three to thirteen percent (Dai et al. 2012; Kwak et al. 2010; Takanashi et al. 2015; Martelli et al. 2009) and was also close to those reported in more recent studies indicating this rate to be three to five percent (Gao et al. 2017; Lin et al. 2017). The decrease in the frequency of *ALK* positivity may be due to the lack of standardisation of methods. In this paper, FISH with a multicolour break-apart *ALK* probe, which is an FDA-approved method, was used to screen for *ALK* gene rearrangements. This method has been reported to be effective in the assessment of *ALK* gene rearrangements in formalin-fixed, paraffin-embedded NSCLC tumour samples (Dai et al. 2012); additionally, newer technologies such as targeted next generation-sequencing have also been suggested as possible methods to be used for screening *ALK* gene rearrangements (Letovanec et al. 2017).

In lung carcinoma, the most common rearrangement of the *ALK* gene is the short arm inversion of chromosome 2, which leads to the formation of the *EML4-ALK* fusion (fusion of the 5' end of the *EML4* gene [located at 2p21] with the 3' end of the *ALK* gene [at 2p23]) (Dai et al. 2012). FISH may also detect other chromosomal rearrangements such as deletions and amplifications due to intratumoral heterogeneity, which should be considered when evaluating results for analysis of *ALK* gene rearrangements. Amplification signals other than those for the *EML4-ALK* fusion are sometimes ignored when evaluating FISH results; however, since *ALK* fusion amplification detected by FISH can be an early sign of resistance in patients undergoing crizotinib therapy (Katayama et al. 2012), these alternate signals should indeed be considered for NSCLC patients undergoing treatment (Camidge et al. 2012; Dai et al. 2012). These data imply that a further study of variant FISH signals may allow the development of new follow-up treatment approaches.

Currently, detection of *ALK/ROS1* rearrangements is based on immunohistochemistry (IHC) or FISH in NSCLC tumour samples. These tests are considered feasible in circulating tumour cells obtained from NSCLC patients. Recent studies have focused to compare *ALK* FISH and IHC analyses of circulating tumour cells via isolation by size of epithelial tumour (ISET) system and most of them showed perfect concordance for *ALK* rearrangements between circulating tumour cells and tumour samples (Ilie et al. 2012; He et al. 2016; Tan et al. 2016). Accordingly, it is

now believed that liquid biopsy may be a promising methodology for patients unable to be invasively biopsied (Wang et al. 2017).

CONCLUSION

The results of this paper suggested that the frequency of *ALK* rearrangement might not differ significantly among different populations and that *ALK* gene rearrangement might be a pathology specific to certain tumour types. To achieve molecular inhibition of *ALK* for targeted therapy of NSCLC patients, *ALK* positivity of tumour is necessary to be confirmed and this is a real-time predictor of benefiting from the therapy. Accordingly, the method of choice is important in detecting the *ALK* gene rearrangements and there is considerable progress in adapting novel technologies to *ALK* testing. However, since real-time polymerase chain reaction and next generation-sequencing based targeted assays are unlikely to detect fusions with novel partners, FDA-approved FISH could be chosen as a stand-alone test.

RECOMMENDATIONS

Given that *ALK* rearrangements provide a target for NSCLC therapy, molecular profiling of lung cancer tumours should be performed for all NSCLC patients in order to ensure that they receive the most effective therapy protocols.

Standardization of the protocol and performing a validated protocol is necessary to obtain more precise results. FISH methodology using FDA-approved Abbott Molecular break-apart probe appears to be a good choice as the screening technique.

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Paper received for publication on December 2017
Paper accepted for publication on February 2018