

Anaplastic lymphoma kinase (ALK) gene alteration in signet ring cell carcinoma of the gastrointestinal tract

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Abstract

Objectives: ALK-EML4 translocation is an established driver aberration in non-small cell lung cancer (NSCLC), with reported predilection for cases with signet ring histology. We assessed the presence of anaplastic lymphoma kinase (ALK) gene rearrangements in signet ring cancers arising in the stomach and colon.

Methods: Histologically confirmed cases of signet ring adenocarcinoma of the stomach or the colon were identified. The presence of the classic ALK and EML4 fusion gene was initially determined by fluorescence *in-situ* hybridization (FISH) technique. Immunohistochemistry (IHC) was performed using two previously validated antibodies, ALK1 clone (1:100; DAKO) and 5A4 (Novocastra, Leica Biosystems) along with positive controls of ALK-translocated lung cancer.

Results: We employed 42 cases of signet ring carcinoma diagnosed between 2001 and 2011; 25 gastric and 17 colon cancer. Median age 63.3 years; male/female 17/25; race, black 47.5%, white 47.5%, others, 5%; stage I, 21.4%; stage II, 31%; stage III, 26.2%; stage IV, 21.4%. One of 42 cases (2.3%) was positive for ALK translocation by FISH using the standard criteria of at least 15% positive cells for the break-apart signal (50–70 cells enumerated per case). Using a less restrictive cut-off of 10% positive cells, 7 cases (16%) were considered possibly positive. None of the ‘possibly positive’ cases was found to harbor ALK translocation by another molecular testing approach (IHC). IHC with two previously validated monoclonal antibodies showed 0 of 42 (0%) cases positive.

Conclusions: ALK gene rearrangement is very rare in gastrointestinal cancers and enrichment strategy focusing on signet ring cell histology did not significantly improve the detection rate.

Keywords: ALK rearrangement, ALK-EML4 fusion, colon, signet ring cell cancer, stomach

Introduction

Colorectal cancer (CRC) and gastric cancers are leading causes of cancer-related mortality in the United States and worldwide [Ferlay *et al.* 2010]. The mortality arising from CRC or gastric cancer is due mainly to metastatic disease, which is not effectively controlled with currently available systemic therapies [Tsuji *et al.* 1997]. The traditional approach to drug development where agents are developed for clinically selected patient group based on tissue or site of origin [Park *et al.* 2004] is beginning to give way to targeted drug

development for molecularly defined patient groups. This approach has been made possible by the improved understanding of the principal molecular mechanisms driving cancer development. As such, agents are now being developed to target patient subgroups with well-validated driver molecular aberration. The development of crizotinib for non-small cell lung cancer (NSCLC) harboring a translocation between the anaplastic lymphoma kinase (*ALK*) and echinoderm microtubule associated protein like 4 (*EML4*) genes is a clear example of this evolving paradigm in cancer

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therapeutics [La Thangue and Kerr, 2011; Roskoski, 2013]. The initial recognition of EML4-ALK rearrangement as a driver aberration in lung cancer was made in cell lines [Soda *et al.* 2007]. The subsequent confirmation of this finding in patient samples from various centers in Asia and the United States established the clinical relevance, leading to clinical development of specific agents against this target. The identification of a subset of patients with NSCLC harboring ALK gene translocation facilitated the successful development of crizotinib as a new treatment option in lung cancer [Ou *et al.* 2011].

Moreover, the unique clinical and pathologic features of ALK-rearranged lung cancer enabled case identification. For instance, ALK positive lung cancers are more likely to be diagnosed in younger age group (<50 years) and patients with no significant history of prior tobacco exposure [Galetta *et al.* 2012]. ALK rearranged lung cancer were also observed to be of mucinous histology and to have the signet ring morphology in some of the reported studies [Rodig *et al.* 2009; Jokoji *et al.* 2010; Popat *et al.* 2012]. There is no known anatomic or biologic mechanism to suggest that ALK gene rearrangement is selective or restricted to lung cancers. Therefore, attempts have been made to characterize other tumor types for the presence of ALK rearrangement as a prelude to testing ALK inhibitors in these tumor types [Grob *et al.* 2012; Krishnamurthy *et al.* 2013; Niu *et al.* 2013].

The incidence of ALK gene alterations in other malignancies such as colorectal or gastric cancer has not been well studied. Two prior studies reported a very low prevalence of EML4-ALK rearrangement in colorectal cancer of 0% (0 of 770) [Bavi *et al.* 2013] and 2.4% (2 of 83 patients) [Lin *et al.* 2009]. These studies, however, did not employ any enrichment strategy to improve the detection rate. We therefore hypothesized that the use of tumor histology as an enrichment strategy will lead to a higher rate of detection and facilitate the development of therapeutic agents for this subset of patients. We therefore decided to evaluate for EML4-ALK translocation in the subset of colon and gastric cancer with signet ring histology. In addition, we employed two different testing platforms to detect dysregulated ALK signaling, which in addition to the well-characterized fusion with EML4, can also result from specific activating mutations, gene amplification or fusion with other partner genes [Mosse *et al.* 2009].

Materials and methods

Patient samples

We reviewed cases of gastric and colorectal cancers treated at Winship Cancer Institute of Emory University between 2001 and 2011 in order to identify eligible cases for the study. The study was approved by the Emory Institution Review Board. A convenient sampling method was used whereby all cases of signet ring adenocarcinoma meeting the stated eligibility criteria during the period under review were included. The salient criteria for inclusion were: a pathologically confirmed diagnosis, availability of adequate tissue samples and reliable records for clinical care and survival. Electronic records of the selected cases of gastric and colorectal cancers treated were reviewed in order to obtain relevant clinical data including: demographics (age, gender, and ethnicity), date of diagnosis, type of treatment and course of disease, family or genetic predisposition, and other potential risk factors. The corresponding paraffin-embedded tissue specimens (FFPE) obtained at the time of original diagnosis were retrieved from the archives of the department of pathology. A total of 90% of the archival samples were suitable for inclusion.

Fluorescence in-situ hybridization Assay

The presence of the classic ALK-EML4 fusion gene was initially determined using the current gold standard break-apart fluorescence *in-situ* hybridization (FISH) test as described previously [Ou *et al.* 2011]. Interphase molecular cytogenetic studies using a commercially available ALK probe (Vysis LSI ALK Dual Color, Break Apart Rearrangement Probe; Abbott Molecular, Abbott Park, IL) were performed on paraffin-embedded sections that were deparaffinized three times in CitriSolv for 10 minutes each, dehydrated twice in 100% ethylic alcohol for 5 minutes. Tissue sections were then transferred to 85°C standard saline citrate (2xSSC) for 15 minutes, and protein was digested with 0.6 mg/ml Proteinase K (Sigma) at 45°C for 16 minutes. After brief washing in 1x phosphate-buffered saline, the slides were sequentially dehydrated in alcohol (70%, 85%, and 100%) and air dried at room temperature. ALK probe was applied to the selected tumor area, which was covered and sealed with rubber cement. Slides were denatured at 85°C for 15 minutes, and probe hybridization was carried out overnight in a humidified chamber at 37°C. Tissue sections were washed in 0.3% NP40/1x SSC at 74°C for 2

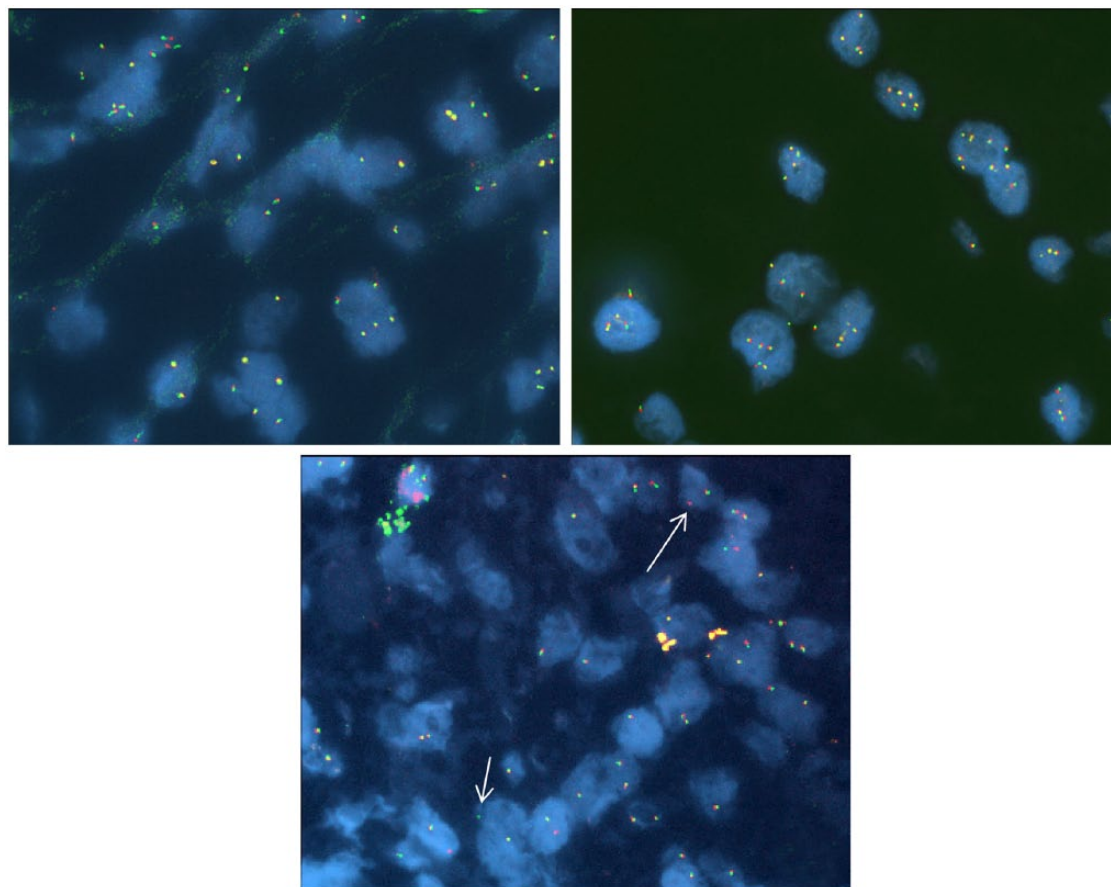


Figure 1. Break-apart fluorescence *in-situ* hybridization (FISH) probe for anaplastic lymphoma kinase (ALK): (1) ALK FISH break-apart probe set showing normal fused red/green signals; (2) ALK FISH break-apart probe set showing high number of copies of fused red/green signals per nucleus; (3) ALK FISH break-apart probe set showing isolated 3'ALK (single red signal - long arrow) and 5' ALK (single green signal - short arrow).

minutes and then washed in 2xSSC at room temperature for 2 minutes. Slides were mounted in VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA) with 1.5 g/ml of 4, 6-diamidino-2-phenylindole. In this test, 5' (red) and 3' (green) fluorescent probes bind to areas upstream and downstream of the rearrangement breakpoint in exon 20 of the ALK gene(19).

When the ALK gene is intact, the red and green signals are closely apposed together and appear fused. They are referred to as a fused signal, sometimes registering as a composite yellow signal (Figure 1). With ALK gene rearrangement, the signals move apart from each other to give the split signal, which is considered positive. We scored a total of 50 to 80 tumor cells per specimens and presence of at least 15% of cells showing separated 3'ALK and 5'ALK signals or single 3'ALK signals classified the specimen as positive for ALK rearrangement [Kwak *et al.* 2010].

Immunohistochemistry

Expression of ALK protein was determined using standard IHC techniques along with positive and negative controls. The commercially available antibodies employed for IHC were previously employed by other groups to determine ALK protein expression in pathologic specimens [Mino-Kenudson *et al.* 2010]. Briefly, following deparaffinization, antigen retrieval was performed using Target Retrieval Solution diluted 1:10 (pH 6). The slides were placed in this solution and heated to 120°C for 4 minutes using Biocare Decloaking Chamber. Afterward, the slides were allowed to cool in hot solution for 20 minutes, and were gently washed in running tap water. The slides were placed in a wash buffer bath (Tris Buffered Saline [TBS] and Tween 20), and endogenous peroxidase blocking was established with 3% H₂O₂ for 5 minutes. The slides were subsequently rinsed in TBS before incubation with primary antibodies ALK1 clone (1:100; DAKO)

Table 1. Patient and tumor characteristics.

		N = 42	%
Gender	Female	25	59.5
	Male	17	40.5
Race	African American	19	47.5
	Other	2	5.0
	White	19	47.5
	Missing	2	-
Histology	Colon	17	40.5
	Gastric	25	59.5
Stage	I	9	21.4
	II	13	31.0
	III	11	26.2
	IV	9	21.4
Age	Overall		
	Mean (SD)	63.2 (12.8)	
	Median (IQR)	63.3 (5)	

SD, standard deviation; IQR, interquartile range.

and ALK Clone 5A4 (Novocastra, Leica Biosystems). Protein expression was assessed jointly by two of the authors (GS; TKO) using previously published standard algorithm: negative staining (0), weak intensity (1+), moderate intensity (2+) and strong intensity (3+). The proportion of the staining cells were also determined and semi-quantitatively expressed as 0 (0%), 1+ (1–25%), 2+ (26–50%), 3+ (51–75%), 4+ (76–100%). Any sample with staining intensity $\geq 2+$ was considered positive for dysregulated ALK signaling.

Statistical analysis

The frequency of ALK dysregulation in gastrointestinal cancer is currently unknown. However, based on the NSCLC data, a 10% prevalence rate was considered interesting enough to pursue further development of a diagnostic or therapeutic intervention. Descriptive statistics such as mean, median, range and standard deviation of the mean score were generated for the histologic results. Inferential statistics were performed as applicable using Student's *t*-test or Mann–Whitney test depending on the distribution of the data. The significance levels were set at 0.05 for all tests. The SAS statistical package V9.3 (SAS Institute,

Inc., Cary, NC) was used for data managements and analyses.

Results

Clinical characteristics

We identified 42 cases of signet ring carcinoma from the cancer registry database; 25 gastric and 17 colon cancers. Table 1 details the baseline demographics and disease characteristics of the identified cases. The median age was 63.3 years with a female-to-male ratio of 1.5. There were equal numbers of African Americans and non-Hispanic white (47.5% each).

ALK rearrangement detection by FISH

There was a single case (gastric adenocarcinoma with signet ring features) out of the 42 cases (2.3%) that was positive for ALK rearrangement by FISH, using the standard criteria of $\geq 15\%$ of cells being positive for the break-apart signal (Figure 2). This was a total gastrectomy specimen and evaluation was based on enumeration of 50–70 cells per case. Using a less restrictive cut-off of 10% positive cells, 7 cases (16%) were considered either positive or 'possibly positive'. Among these, there were 6 of 25 (26%) gastric cancer and 1 of 17 (6%) colon cancer cases.

ALK expression detection by IHC

Using an intensity score of 1 or greater to indicate positive ALK expression, we assessed 0 of 42 cases (0%) as positive. The two cases of lung cancer with known ALK-EML4 translocation that served as positive controls both stained positive with a stronger staining intensity obtained with the Leica antibody in one of the two cases.

Discussion

Signet ring cell carcinoma (SRC) usually arises in the glandular epithelium of the gastrointestinal tract. This histologic subtype is reported in up to 26% of gastric cancers [Taghavi *et al.* 2012] and in about 2% of colorectal carcinomas [Kwon *et al.* 2014]. In comparison with adenocarcinoma, SRC arising in the stomach or the colorectal site is more frequent in younger patients, in women and more likely to present at a more advanced stage of disease. Coupled with the predilection for gastric SRC to involve the body and distal stomach and for colonic SRC to present as a right-sided colon cancer, we expect that this

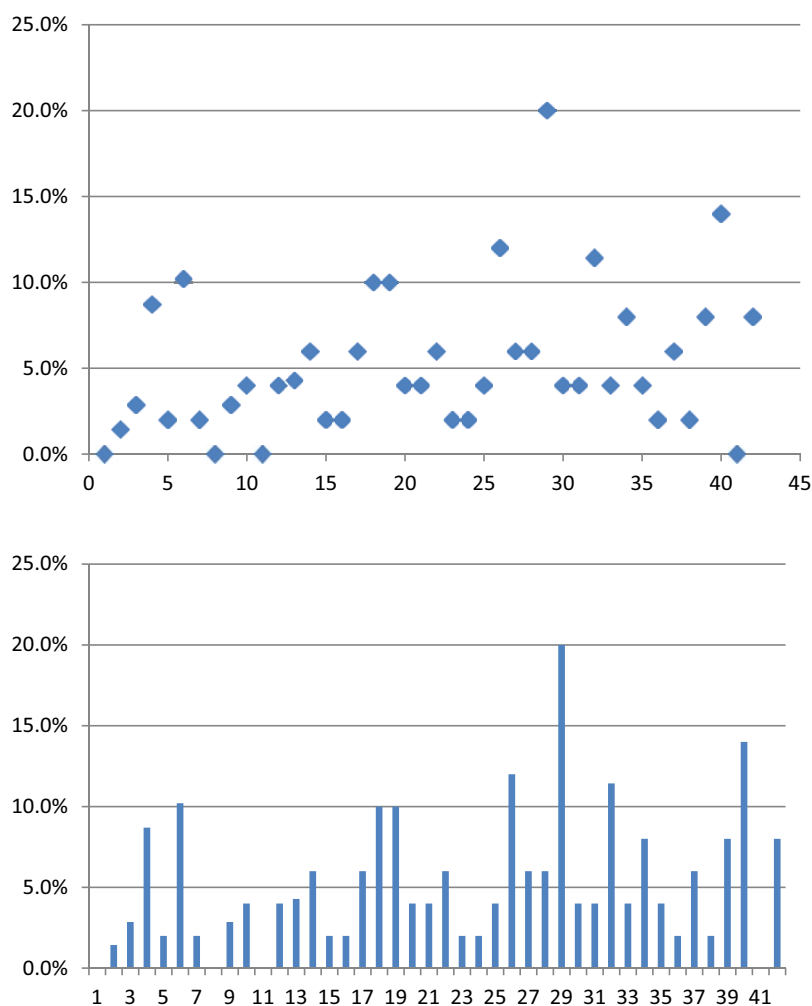


Figure 2. Scatterplot and histogram showing percentage of cells with non-fused break-apart signals detected by fluorescence *in-situ* hybridization (FISH) in each of the two cases of colon and gastric adenocarcinoma employed in this study. There was a single case out of the 42 cases (2.3%) that was positive for anaplastic lymphoma kinase (ALK) rearrangement by FISH using the standard criteria of $\geq 15\%$ of cells being positive for the break-apart signal. Using a less-restrictive cut-off of 10% positive cells, 7 cases (16%) were considered either positive or possibly positive.

subtype of cancer will manifest a distinct biology to match its unique anatomy and histology. Based on the experience in lung cancer where ALK rearranged tumors were more likely to have signet ring features [Shaw *et al.* 2009], we decided to use tumor histology as an enrichment strategy to facilitate the detection of this subtype of cancer in the expectation that our finding will inform targeted therapeutic strategy leading to a new option for managing SRC of the gastrointestinal tract.

The presence of rearranged ALK gene using IHC or break-apart FISH was required prior to enrollment into the early phase I trial of crizotinib (A8081005). The test was performed at various commercial laboratories prior to the accelerated

approval of crizotinib in August 2011. However, FDA approval of crizotinib was based on the presence of ALK translocation in at least 15% of scored cells using FISH as the standard companion diagnostic test [Monjour *et al.* 1988]. The ALK signaling pathway may be dysregulated due to various mechanisms (activating mutations, gene amplification, translocations). However, the standard FISH test detects only gene rearrangements regardless of the specific fusion companion or breakpoint variant. This could lead to false-negative results if there are complex rearrangements not detectable by the selected FISH probe. Indeed, there was no correlation between maximal tumor reduction in patients treated with crizotinib and the proportion of cells harboring the

classic gene alteration within the tumor sample [Camidge *et al.* 2012]. This well-described limitation of the FISH assay [Weickhardt *et al.* 2013] could have contributed to the low frequency of ALK positive cases in our series using the previously established cutoff point of 15% from lung cancer patients. The biological significance of a different cut-point of 10% could not be assessed in this retrospective dataset.

We employed IHC as an alternative detection platform to address this limitation of FISH assay since it can detect increased ALK expression that result from different mechanisms of genetic alteration including activating mutations, amplification and translocation [Yi *et al.* 2011]. With a reported sensitivity comparable with FISH and specificity approaching 100%, IHC is a viable and possibly superior alternative to FISH but requires rigorous tissue processing protocols [Mino-Kenudson *et al.* 2010]. We failed to detect increased ALK expression by IHC in any of the 42 cases tested in this study, including the single case that was positive by FISH. The failure to show ALK overexpression in the FISH positive case could be due to the cohesive nature of signet ring cancers leading to relatively scanty amount of cellular material against a broad infiltrative stroma [Borger *et al.* 2007].

Our study used two different platforms (performed in a blinded manner), namely IHC and FISH to carefully study the incidence of *ALK* gene alterations in gastric and colon SRCs. These complementary techniques demonstrated that *ALK* gene alteration is a very rare genetic event in SRC. Nonetheless, the identification of a single case both by FISH indicates the potential role of this genetic driver in a small subset of SRC. Our results have further extended the finding by other workers showing a very low frequency of *ALK* gene alteration in colon cancer. Our detection rate of 4.7% by FISH is comparable to the rate reported by other investigators ranging between 3% and 5% in various gastrointestinal cancer [Aisner *et al.* 2014]. An enrichment strategy focusing only on SRC histology did not increase the detection rate. Moreover, our inability on IHC to observe increased ALK expression in the single case detected by FISH suggests that caution should be exercised when employing IHC as a surrogate standalone platform for *ALK* translocation testing, at least in SRC.

In conclusion, *ALK* gene rearrangement in gastrointestinal cancers is very rare and enrichment strategy focusing on signet ring cell histology did

not significantly improve the detection rate. The failure to detect higher number of cases even with the use of different testing platforms further supports the previous work that reported rare occurrence of *ALK* translocation in gastrointestinal cancers. Further studies are needed to identify prognostic and therapeutic molecular targets in SRC of gastric and colorectal cancers.

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Conflict of interest statement

The authors declare no conflict of interest in publishing this article.

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