Concordance of anaplastic lymphoma kinase (ALK) gene rearrangements between circulating tumor cells and tumor in non-small cell lung cancer

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ABSTRACT

Anaplastic lymphoma kinase (ALK) gene rearrangement in non-small cell lung cancer (NSCLC) is routinely evaluated by fluorescent in-situ hybridization (FISH) testing on biopsy tissues. Testing can be challenging however, when suitable tissue samples are unavailable. We examined the relevance of circulating tumor cells (CTC) as a surrogate for biopsy-based FISH testing. We assessed paired tumor and CTC samples from patients with ALK rearranged lung cancer (n = 14), ALK-negative lung cancer (n = 12), and healthy controls (n = 5) to derive discriminant CTC counts, and to compare ALK rearrangement patterns. Blood samples were enriched for CTCs to be used for ALK FISH testing. ALK-positive CTCs counts were higher in ALK-positive NSCLC patients (3–15 cells/1.88 mL of blood) compared with ALK-negative NSCLC patients and healthy donors (0–2 cells/1.88 mL of blood). The latter range was validated as the ‘false positive’ cutoff for ALK FISH testing of CTCs. ALK FISH signal patterns observed on tumor biopsies were recapitulated in CTCs in all cases. Sequential CTC counts in an index case of lung cancer with no evaluable tumor tissue treated with crizotinib showed six, three and eleven ALK-positive CTCs per 1.88 mL blood at baseline, partial response and post-progression time points, respectively. Furthermore, ALK FISH rearrangement suggestive of gene copy number increase was observed in CTCs following progression. Recapitulation of ALK rearrangement patterns in the tumor on CTCs, suggested that CTCs might be used to complement tissue-based ALK testing in NSCLC to guide ALK-targeted therapy when suitable tissue biopsy samples are unavailable for testing.

INTRODUCTION

Lung cancer accounts for about 13% of all cancer diagnoses and remains the leading cause of death by cancer in the world [1], with almost 70% of patients diagnosed with locally advanced or metastatic disease at presentation [1, 2]. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all cases of lung cancer and is associated with poor prognosis [2]. The 5-year overall survival rate for NSCLC across all stages is only 21% and is even lower (~5%) for stages IIIIB and IV [1, 3].
Oncogenic ‘driver mutations’ have now been identified in various subsets of NSCLC [4-6]. Of these, somatic mutations in epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) [5-7] are the most frequently described. In 2007, researchers identified the presence of a chimeric ALK protein with fibroblast-transforming properties that was formed following fusion of the echinoderm microtubule-associated protein-like 4 (EML4) and ALK genes [6]. EML4-ALK subverts intracellular signaling pathways to promote tumor cell survival and growth [8]. The overall incidence of ALK gene rearrangement in NSCLC ranges between 0.4% and 13.4%, and is similar in both Asian and Western populations [9]. This discovery resulted in the accelerated development and approval by the U.S. Food and Drug Administration (FDA) of the ALK-targeting tyrosine kinase inhibitors (TKIs) crizotinib (Xalkori™, Pfizer, New York, USA) in 2011, and ceritinib (Zykadia™, Novartis, Basel, Switzerland) in 2014 to treat patients with metastatic NSCLC who express the abnormal ALK gene [10, 11].

The true therapeutic benefit of novel molecules targeting the mutant ALK fusion protein in NSCLC relies on identifying the right patient population for treatment, and on detecting the emergence of tumor resistance. The American Society of Clinical Oncology (ASCO) endorsed the joint College of American Pathologists (CAP)/International Association for the Study of Lung Cancer (IASLC)/Association for Molecular Pathology (AMP) clinical practice guideline on EGFR and ALK molecular testing for patients with lung cancer, which holds that an clinical practice guideline on EGFR and anaplastic lymphoma kinase (ALK) drivers, somatic mutations in epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) (IASLC)/Association for Molecular Pathology (AMP) clinical practice guideline on EGFR and ALK molecular testing for patients with lung cancer, which holds that an clinical practice guideline on EGFR and anaplastic lymphoma kinase (ALK) dual-labeled break-apart probes is the preferred testing model to examine the concordance patterns between CTCs and tumor tissue, and to determine whether CTCs were reproducibly detectable in circulation. We further explored the potential use of CTCs in lung cancer, as a surrogate for molecular testing of the primary tumor for ALK gene rearrangement.

RESULTS

Study group

We prospectively recruited 27, mostly late-stage NSCLC patients, 14 of whom had ALK-rearranged and 12 had wild-type ALK, determined from the initial biopsy diagnoses. One patient in the cohort, who was not from Singapore, had an unknown ALK status due to incomplete referral records. Sixty percent of patients were males. All ALK-positive patients were non-smokers. Five healthy donors (three males and two females), aged between 18-55 years old with no history of cancer, were also recruited into the control cohort. As ALK translocation in NSCLC
patients is strongly correlated with a non-smoker or light smoker status [29], these healthy donors were non-smokers. The clinicopathologic features of the study group are summarized in Table 1.

### Histopathological analysis of tumor tissues

In the ALK-positive group, three out of 14 tumor tissues exhibited morphology that is associated with ALK rearrangements [30]. Histological preparations showed solid adenocarcinoma with signet ring cells (Figure 1A and 1B), and cribriform adenocarcinoma with focal squamoid cells (not shown). Immunohistochemical (IHC) studies showed strong and diffuse nuclear reaction for Thyroid Transcription Factor-1 (TTF-1). This finding confirmed the diagnosis of adenocarcinoma of lung origin in this particular setting (Figure 1C). Some tumors also showed focal reaction to periodic acid-Schiff with diastase (PAS-D) within mucin vacuoles, which is a general feature of adenocarcinomas, as opposed to squamous cell carcinomas (Figure 1D) [30].

### Concordance in ALK rearrangement pattern between CTCs and tumor

Following FISH testing on all tumor samples in the cohort, it was found that ALK-positive tumors harbored ALK rearrangements with various patterns of abnormality (Table 2). The majority of the tumor samples harbored the one fusion (F) and one split orange (R) and green (G) signal (Figure 2A). The tumor from Patient P5 presented various ALK rearrangement patterns such as 1F1R1G, 2F1R, 2F2R, 1F1R and 1F2R.

FISH testing was subsequently performed on CTCs that were enriched and isolated from the matched blood samples. Data showed that ALK rearrangement patterns (majority 1F1R1G) observed in primary tumor tissues were recapitulated on most of the ALK-positive CTCs, giving an overall concordance rate of over 90% based on the 1F1R1G fusion pattern (Table 2). In Patient P5 (Table 2), the CTCs were able to recapitulate three out of five ALK rearrangement patterns observed in the tumor tissue.

We further observed an overexpression of vimentin in the tumor samples, along with the control bronchiolar epithelium (Figure 2B). However, loss of E-cadherin was not obvious in these samples.

The number of ALK-positive rearranged CTCs

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**Table 1: Clinicopathological characteristics of patients enrolled in this study**

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years</strong></td>
<td>32–76</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>16 (59.3%)</td>
</tr>
<tr>
<td>Female</td>
<td>11 (40.7%)</td>
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<tr>
<td><strong>Smoking history</strong></td>
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</tr>
<tr>
<td>Non-smoker</td>
<td>16 (59.3%)</td>
</tr>
<tr>
<td>Smoker</td>
<td>5 (18.5%)</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>5 (18.5%)</td>
</tr>
<tr>
<td>No info</td>
<td>1 (3.7%)</td>
</tr>
<tr>
<td><strong>Clinical staging</strong></td>
<td></td>
</tr>
<tr>
<td>IB</td>
<td>1 (3.7%)</td>
</tr>
<tr>
<td>IIIA</td>
<td>1 (3.7%)</td>
</tr>
<tr>
<td>IIIB</td>
<td>2 (7.4%)</td>
</tr>
<tr>
<td>IV</td>
<td>23 (85.2%)</td>
</tr>
<tr>
<td><strong>Histological subtype</strong></td>
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<tr>
<td>ALK-positive</td>
<td>14 (51.9%)</td>
</tr>
<tr>
<td>Adenocarcinoma (NSCLC)</td>
<td>11 (40.7%)</td>
</tr>
<tr>
<td>Unknown subtype (NSCLC)</td>
<td>3 (11.1%)</td>
</tr>
<tr>
<td>ALK-negative</td>
<td>12 (44.4%)</td>
</tr>
<tr>
<td>Adenocarcinoma (NSCLC)</td>
<td>5 (18.5%)</td>
</tr>
<tr>
<td>Unknown subtype (NSCLC)</td>
<td>7 (25.9%)</td>
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<tr>
<td>ALK status unknown</td>
<td>1 (3.7%)</td>
</tr>
<tr>
<td>Adenocarcinoma (NSCLC)</td>
<td>1 (3.7%)</td>
</tr>
</tbody>
</table>

Abbreviations: ALK, anaplastic lymphoma kinase; NSCLC, non-small cell lung cancer
### Table 2: Concordance of ALK rearrangement patterns between CTC and tumor in patients with ALK-positive NSCLC

<table>
<thead>
<tr>
<th>Case number of patients with ALK-positive NSCLC</th>
<th>ALK rearranged/ total cells scored (Tumor)</th>
<th>ALK rearrangement patterns (% of tumor cells observed with respective patterns)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tumor</td>
</tr>
<tr>
<td>P1</td>
<td>61/100</td>
<td>1F1R1G (100%)</td>
</tr>
<tr>
<td>P2</td>
<td>45/100</td>
<td>1F1R1G (100%)</td>
</tr>
<tr>
<td>P3</td>
<td>79/100</td>
<td>1F1R1G (100%)</td>
</tr>
<tr>
<td>P4</td>
<td>30/100</td>
<td>1F1R1G (100%)</td>
</tr>
<tr>
<td>P5</td>
<td>61/100</td>
<td>1F1R1G (4.9%) 2F1R (34.4%) 2F2R (3.3%) 1F1R (49.2%) 1F2R (8.2%)</td>
</tr>
<tr>
<td>P6</td>
<td>72/100</td>
<td>1F1R1G (29.2%) 1F1R (70.8%)</td>
</tr>
<tr>
<td>P7</td>
<td>81/100</td>
<td>1F1R (100%)</td>
</tr>
<tr>
<td>P8</td>
<td>55/100</td>
<td>1F1R1G (100%)</td>
</tr>
<tr>
<td>P9</td>
<td>77/100</td>
<td>1F1R (31.2%) 1F1R1G (68.8%)</td>
</tr>
<tr>
<td>P10</td>
<td>100/100</td>
<td>1F1R (100%)</td>
</tr>
<tr>
<td>P11</td>
<td>62/100</td>
<td>1F1R1G (100%)</td>
</tr>
<tr>
<td>P12</td>
<td>77/100</td>
<td>1F1R1G (100%)</td>
</tr>
<tr>
<td>P13</td>
<td>45/100</td>
<td>1F1R1G (100%)</td>
</tr>
<tr>
<td>P14</td>
<td>Not available</td>
<td>Not available</td>
</tr>
</tbody>
</table>

Abbreviations: ALK, anaplastic lymphoma kinase; CTC, circulating tumor cells; NSCLC, non-small cell lung cancer.

**Figure 1: Representative appearance of NSCLC adenocarcinoma with signet ring cells features.**

- **A.** H&E stain showing solid nests of tumor cells
- **B.** Solid with signet ring cells (arrow)
- **C.** Thyroid transcription factor-1 (TTF-1) IHC stain showing strong nuclear reaction in the signet ring cells.
- **D.** Solid tumor showing focal positive reaction for (PAS-D) within mucin vacuoles (arrow).
Figure 2: High concordance of *ALK* FISH rearrangements patterns between CTCs and tumors in NSCLC adenocarcinoma patients. A. Representative *ALK* FISH rearrangement patterns in CTCs and tumors showing 1F1R1G rearrangement patterns. Yellow, red and green arrows represent fusion (F), orange (R) and green (G) fluorescent signals. B. Representative vimentin (upper panel) and E-cadherin (lower panel) IHC in tumor and control bronchiolar epithelium (black arrow).
retrieved from ALK-positive patients was significantly enriched compared with ALK-negative patients \( (p < 0.0001) \) and healthy donors \( (p = 0.0003) \) (Figure 3).

**Establishment and validation of ALK break-apart probes cutoff in ALK-negative samples**

ALK testing by FISH in NSCLC tumor tissues without ALK rearrangement may detect rearrangement-positive patterns (i.e. split patterns or isolated 3’ patterns) in a fraction of cells [31-33], likely because of truncation artefact caused by tissue sectioning, or perhaps a stochastic genomic alteration that does not indicate a specific gene fusion. ALK FISH testing in formalin-fixed, paraffin-embedded (FFPE) NSCLC tumor tissues has a ‘false positive’ cutoff value of 15% to allow for the best separation between ALK-rearranged and ALK wild-type cells [31, 33]. However, it is not possible to apply this guideline in the ALK FISH testing on CTCs because the number of CTCs in any given blood sample would be too low.

In our study, we established and validated the ‘false positive’ cutoff for ALK FISH in CTCs using 12 blood samples from NSCLC ALK-negative patients and five blood samples from healthy donors (Supplementary Data). Results from the ALK-negative NSCLC cohort scored a median of two or less positive cells (range 0-2 cells/1.88 mL blood). The result concurred with the numbers observed for healthy blood samples. In fact, no statistical difference in ALK-positive cell counts was observed between ALK-negative NSCLC cohort and healthy donors \( (p = 0.0973) \) (Figure 3). This data established the ‘false-positive’ cutoff for ALK break-apart probes in CTCs at \( \leq \) two cells per 1.88 mL blood.

**Potential clinical applications**

Sequential CTC enumeration and FISH was performed on blood samples from a patient with no accessible tissue for ALK FISH testing. The index case was a never smoker male diagnosed with NSCLC. A transthoracic needle aspiration biopsy was performed on the right hilar mass to obtain a specimen for histological analysis. The hematoxylin and eosin (H&E) stain showed one small cluster of NSCLC cells with strong nuclear reaction for TTF-1 favoring adenocarcinoma. Unfortunately, his diagnostic tissue was exhausted and no further molecular profiling could be performed. He did not

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**Figure 3: Number of cells with ALK rearrangements in ALK-positive NSCLC patients is significantly higher compared to ALK-negative and healthy donors.** Graph represents statistical analyses of the data on Table S1 using the non-parametric two-tailed \( t \)-test. NS represents not significant while \( p \) value \( <0.05 \) were considered significant.
respond to EGFR tyrosine kinase inhibitor (TKI) therapy and had a short duration of response to pemetrexed and cisplatin. Re-biopsy of the lung and liver tumors was considered but the patient declined due to the risk of bleeding. He consented to blood sampling instead; the sample was subsequently processed as described in the Methods section.

At baseline, six CTCs displaying a 1F1R1G pattern were isolated and met the necessary cutoffs for ALK-positivity (Figure 4A). A trial of crizotinib was commenced. Confirmatory scans done 3 months after completion of treatment demonstrated good partial response in the liver and minor response in the primary lung tumor, based on RECIST criteria (Figure 4A). Sequential CTC counts dropped to three cells displaying the similar ALK rearranged pattern as the baseline. He continued on crizotinib but unfortunately, his disease progressed in the liver and the brain 5 months after treatment initiation (Figure 4A). A post-progression blood sample showed additional ALK rearrangement patterns present in his CTCs, which differed from the baseline patterns. New ALK rearrangement patterns such as 2R2G and 1F1R appeared, in addition to 1F1R1G, which was previously present (Figure 4B). The number of ALK-positive CTCs also increased from three to eleven CTCs per 1.88 mL of blood post-progression.

**DISCUSSION**

We successfully captured CTCs using an antibody-independent CTC isolation system. CTCs were enriched from the blood samples collected from 27 NSCLC patients, 14 of whom were ALK-positive. Three of the cases exhibited solid with signet ring cells pattern associated with ALK positivity [34-37]. Overall, CTCs isolated from the ALK-positive patient cohort were above detectable levels, even among previously treated patients.

The presence of ALK rearrangement in CTCs was previously analyzed and reported by French groups using the Isolation by Size of Epithelial Tumor (ISET) system,

![Figure 4: An index case suggests that ALK-rearranged CTCs could have clinical application as a diagnostic biomarker to monitor crizotinib treatment and response.](image-url)

**Figure 4:** An index case suggests that ALK-rearranged CTCs could have clinical application as a diagnostic biomarker to monitor crizotinib treatment and response. A. CT scan taken at baseline, partial response and progression time points showing presence of metastatic tumor in liver (arrow). CTC counts and ALK rearrangement patterns for each time point is indicated in the lower panel. B. Representative images showing 1F1R1G, 2R2G and 1F1R ALK rearrangement patterns following progression on crizotinib treatment. Yellow, red and green arrows represent fusion (F), orange (R) and green (G) fluorescent signals.
which is also an antibody-free system (Rarecells, Paris, France) [22, 23]. They characterized 18 ALK-positive lung tumor and CTC samples showing high concordance in ALK rearrangement among European patient cohort. In agreement with our data reported in this study, ‘false-positive’ signals were similarly observed on CTCs from ALK-negative samples and a 4 cell/mL cutoff was eventually established [22, 38].

In a similarly designed study, Pailler et al. [22] described high concordance in ALK rearrangement patterns between the CTC and tumor biopsies in 18 ALK-positive and 14 ALK-negative patients with metastatic NSCLC. This percentage of concordance is in agreement with our own results. They reported that all ALK-positive NSCLC patients in their cohort had 4 or more ALK-rearranged CTCs per mL of blood. The study did not include healthy donors to establish a ‘false positive’ cutoff for ALK FISH testing of CTCs. They further reported that CTCs harboring the 1F1R1G ALK rearrangement patterns is associated with epithelial-mesenchymal transition (EMT) phenotype [22].

Another study had reported that the EMT phenotype (represented by loss of E-cadherin and expression of vimentin) was more common in ALK-rearranged tumors than other genotypes (38.9%, 19.1%, 26.9% and 14.6% of ALK-rearranged, EGFR-mutated, K-ras mutated and triple negative tumors, respectively; p = 0.015) [39]. Separately, expression of vimentin alone was detected in 49.30% of ALK-rearranged tumors while loss of E-cadherin was detected in 71.30% [39]. In our study, we also observed an overexpression of vimentin in the tumor samples in comparison with the control bronchial epithelial tissue. However, the loss of E-cadherin was not obvious in our tumor samples, which suggested that some of the tumor cells retained their epithelial characteristics within a heterogeneous population of cells. The predominance of this particular ALK rearrangement pattern in our patient’s CTCs is therefore consistent with the observation above suggesting that these tumors and their CTCs may be favoring the EMT pathway.

This study further highlights the utility of antibody-independent microfluidic isolation systems for the isolation and downstream characterization of CTCs compared with immunomagnetic antibody-dependent systems. While the numbers of CTCs isolated here are small and may present substrate limitations to downstream characterization of CTC, it should be noted that the current numbers were derived from <2 mL of blood, as opposed to existing systems which use up to 7.5 mL of blood or more. In addition, we have previously demonstrated that there is an association between CTC number and the volume of blood processed [19]. This suggests a limitation that can be easily overcome.

The index case presented here raises the possibility that CTC enumeration based on ALK FISH may be associated with treatment response with crizotinib by imaging. The appearance of additional ALK rearrangement patterns following progression with crizotinib treatment exhibited a double split in both ALK alleles giving rise to the 2R2G ALK rearrangement pattern. The additional copy of the oncogenic ALK may have contributed to disease progression despite treatment with an ALK inhibitor. This observation is worthy of further inquiry, because while the presence of ALK copy number gain is correlated with crizotinib resistance, as previously reported by Doebele et al. [40], and in vitro studies have identified potential resistance mutations in the ALK gene, for example L1196M, G1269A, S1206Y and G1202R [40, 41], limited analysis of post-progression biopsies of tumors from a phase 1 study of LDK378 suggested that these secondary resistance mutations or gene amplification do not account for a majority of resistance cases [11]. Hence, further work with paired re-biopsies and sequential CTC collection may assist understanding of resistance mechanisms in ALK-driven tumors.

Conclusions drawn from our study are limited by its relative small patient population, as was Pallier’s study [22]. Nonetheless, the converging trend of both studies’ findings is indicative of the utility and potential of CTCs as an alternate target of ALK testing in lung cancer and informs the development of CTC-based technology. More importantly, these studies provide the basis for subsequent, large-scale validation studies.

In summary, high concordance of ALK rearrangement patterns in CTCs and tumors as assessed by ALK FISH testing indicates that CTCs may have utility as a non-invasive surrogate diagnostic tool and may be useful in the longitudinal follow-up for resistance profiling. The availability of a non-invasive tool would improve efforts to guide ALK-rearranged targeted treatment in NSCLC, especially in cases without tissue availability. Further efforts at downstream CTC characterization and culture following enrichment are ongoing.

MATERIALS AND METHODS

Patient recruitment and blood samples

Patients with confirmed NSCLC were recruited into this trial. They were naïve for ALK-targeted TKI treatment, but may have received other forms of chemotherapy. Once informed consent was secured from these patients, their blood samples were processed for CTC analysis. The clinical sample collection protocols were reviewed and approved by SingHealth Centralised Institutional Review Board. Clinicopathological information was also recorded for these patients. Blood samples from healthy donors were used as controls in this study.
Histopathology

H&E was done using the Leica ST5010 XL automated stainer (Leica Biosystems, Wetzlar, Germany) while periodic PAS-D staining was done using the Ventana BenchMark Special Stains automated stainer (Ventana Medical Systems Inc, Tucson, Arizona, USA), following the respective standard protocols. Histological diagnoses were made based on the World Health Organization (WHO) classification [42]. IHC labeling was performed on the Ventana BenchMark Ultra autostainer (Ventana Medical Systems Inc, Tucson, Arizona, USA) using the UltraView detection kit and proprietary Standard CC1 (SC1) pre-treatment sets. The antibodies used with their dilution and pre-treatments were as follows: TTF-1 (Novacastra NCL-TTF-1, clone SPT24, SC1, dilution 1:30), vimentin (DAKO M0725, clone V9, SC1, 1:100) and E-cadherin (Dako M3612, clone MCH-38, SC1, 1:30) antibodies. Histopathology data was reviewed by pathologists who had been accredited by the College of American Pathologists (CAP).

CTC enrichment

Peripheral blood was collected using K2 EDTA vacutainer® blood collection tube (BD, Singapore) and processed within 24 hours. Subsequently, 7.5 mL of whole blood was incubated with red blood cell (RBC) lysis buffer (G-Biosciences, USA) according to manufacturer’s recommendations. Lysed RBCs in the supernatant were discarded after centrifugation. Remaining cell pellet containing CTCs was resuspended in ClearCell® resuspension buffer prior to CTC enrichment using the ClearCell® FX system (Clearbridge BioMedics, Singapore), according to manufacturer’s instruction.

The ClearCell® FX system is an automated CTC enrichment system driven by the CTChip® FR1, a microfluidic biochip to isolate CTCs based on size, deformability and inertia. The isolation principle takes advantage of the inherent Dean vortex flows present in curvilinear channels for CTC enrichment, termed Dean Flow Fractionation (DFF) [43]. The enriched CTC sample output was equally divided into four portions.

Fluorescent in-situ hybridization

Four μm thick FFPE tumor tissue sections were mounted on positively charged slides and deparaffinized. FISH was subsequently performed using the US FDA-approved Vysis ALK Break Apart FISH Probe Kit (Abbott Molecular, Abbott Park, Des Plaines, IL, USA). The 5’ ALK probe was labeled with SpectrumGreen™ (G) and the 3’ ALK probe with SpectrumOrange™ (R). ALK FISH for FFPE tissues were considered positive if at least 15 % of the tumor cells showed abnormal break apart signals as detailed in the IVD Vysis ALK Break Apart FISH Probe Kit and by Camidge et al. [33]. A cell is interpreted as having a split pattern (ALK-positive) when the 5’ (G) and 3’ (R) signals are separated by two or more signal diameters. Cells lacking both fluorescent signals were not evaluated.

One portion (one-quarter) of the enriched CTCs was fixed in Shandon CytoSpin™ Collection Fluid (ThermoFisher Scientific, USA) overnight at 4°C. The sample was deposited onto positively charged glass slides by cytopsin (800 rpm, 5 mins). All the cells on the slides were analyzed for the ALK break-apart signal at 1000X magnification. The scorers analyzing the ALK break-apart signal on CTCs were blinded to the ALK rearrangement patterns on the tumor samples, as well as whether the cell isolated was from patients or healthy controls.

The remaining three portions of the enriched CTCs were stored at 4°C under validated conditions for future molecular testing.

Data analysis

Statistical analyses of the data were performed in GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA). A non-parametric two-tailed, t-test (Mann-Whitney) was used for computing statistical significances. p value of less than 0.05 were considered significant.

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CONFLICTS OF INTEREST

Chwee Teck Lim is an advisor with Clearbridge Biomedics, Singapore. Man Chun Leong is an employee of Clearbridge Biomedics, Singapore. All other authors declare that they do not have any conflicts.
Editorial note

This paper has been accepted based in part on peer-review conducted by another journal and the authors’ response and revisions as well as expedited peer-review in Oncotarget.

REFERENCES


