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Identification of circulating tumor cells with EML4-ALK translocation using fluorescence in situ hybridization in advanced ALK-positive patients with lung cancer

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Abstract
The evaluation of ALK rearrangement in non-small-cell lung cancer (NSCLC) is a significant tool when considering chemotherapy. It is not always possible to perform a tumor biopsy in patients. We suggest isolation and culturing of circulating tumor cells (CTCs) as an alternative to tumor biopsies for the diagnosis of ALK rearrangement. From 22 patients with NSCLC harboring ALK rearrangement, blood was collected and was divided into two parts: one for immunofluorescence staining and the other for culture. Both samples were processed by size-based filtration, and cultured CTCs were analyzed for EML4-ALK translocation by fluorescence in situ hybridization. EpCAM positive and ALK rearrangement CTCs were detected. Therefore, we suggest that the CTCs can be used as an alternative method to tissue biopsy for diagnosing ALK rearrangement. In addition, this method may have clinical applications including serial blood sampling for the development of personalized cancer therapy based on individual genomic information.

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Dear Editor:

Please find enclosed our manuscript entitled "Identification of circulating tumor cells with EML4-ALK translocation using fluorescence in situ hybridization in advanced ALK-positive patients with lung cancer" which we request you to consider for publication as an ***Original Article in oncology reports***

In this paper, we report on the evaluation of ALK rearrangement status on circulating tumor cells (CTCs). We successfully enriched and cultured circulating tumor cells (CTCs) obtained from liquid biopsies and evaluated the ALK rearrangement in these cells. This method represents a suitable, non-invasive alternative to tumor biopsies and will be of interest to clinical oncologists and physicians who are interested in using CTCs in clinical settings.

We confirm that this work is original and has not been published or currently is under consideration for publication elsewhere.

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Thank you for your consideration.

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1 Identification of circulating tumor cells with EML4-ALK translocation using fluorescence *in*
2 *situ* hybridization in advanced ALK-positive patients with lung cancer

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11 **Key words**
12 Lung cancer, culturing circulating tumor cells, EML4-ALK translocation, fluorescence in situ
13 hybridization

14 **Running title**
15 Young Hun Kim et al: DETECTION OF ALK REARRAGNE IN THE CULTURED
16 CIRCULATING TUMOR CELLS

17 **abstract**

18 The evaluation of ALK rearrangement in non-small-cell lung cancer (NSCLC) is a
19 significant tool when considering chemotherapy. It is not always possible to perform a tumor
20 biopsy in patients. We suggest isolation and culturing of circulating tumor cells (CTCs) as an
21 alternative to tumor biopsies for the diagnosis of ALK rearrangement. From 22 patients with
22 NSCLC harboring ALK rearrangement, blood was collected and was divided into two parts:
23 one for immunofluorescence staining and the other for culture. Both samples were processed
24 by size-based filtration, and cultured CTCs were analyzed for EML4-ALK translocation by
25 fluorescence *in situ* hybridization. EpCAM positive and ALK rearrangement CTCs were
26 detected. Therefore, we suggest that the CTCs can be used as an alternative method to tissue
27 biopsy for diagnosing ALK rearrangement. In addition, this method may have clinical
28 applications including serial blood sampling for the development of personalized cancer
29 therapy based on individual genomic information.

30 **Introduction**

31 Lung cancer is the most common type of cancer and is the leading cause of
32 cancer-related mortality worldwide; non-small-cell lung cancer (NSCLC) accounts for 80%
33 of lung cancers . *EGFR* mutations have been detected in 10%–35% of patients with NSCLC,
34 and tumors with *EGFR* mutations are sensitive to treatment with EGFR tyrosine kinase
35 inhibitors (TKIs) . ALK rearrangement is another distinct subtype of lung cancer and is
36 present in 2%–7% of NSCLC . Such tumors display a remarkable sensitivity to ALK
37 inhibitors, such as crizotinib and ceritinib . Most patients with NSCLC with ALK
38 translocations are diagnosed in advanced stages of the disease . Thus, it is usually difficult to
39 obtain the tumor tissue required for diagnosing ALK using fluorescence *in situ* hybridization
40 (FISH), which is a gold standard for diagnosis of ALK translocation. In addition, second
41 biopsies have recently become necessary to evaluate the mutation status of the remaining
42 tumor and to monitor the treatment responses . However, tumor tissue biopsy has its
43 limitations; it is unrepeatable and invasive .

44 Recently, circulating tumor cells (CTCs) were detected in the blood of patients with
45 lung cancer . These CTCs have been utilized for cancer diagnosis and genetic evaluation .
46 CTC isolation technologies are divided into two categories: one is based on the biological
47 properties (cell surface marker proteins) and the other is based on the physical properties
48 (size, deformability, density, and electric charge) . However, the isolation of CTCs remains
49 challenging because of their low sensitivity. Although RT–PCR and qPCR have been used
50 for molecular characterization of CTCs, it has been technologically difficult to detect RNA

51 markers in CTCs. Cytogen's CTC enrichment and culture platform was developed for
52 obtaining sufficient amounts of CTCs to be used for FISH and for genetic analyses, such as
53 genomics and transcriptomics .

54 Here, we investigated whether CTCs can be used to detect ALK rearrangement using
55 FISH. Because a limited number of CTCs can be obtained, we developed a CTC culture
56 method to obtain sufficient CTCs for FISH analysis. In addition, we evaluated the
57 effectiveness of ALK detection by FISH using cultured CTCs.

58 **Materials and methods**

59 Patients and samples

60 This study was approved by the Institutional Review Board of The Catholic
61 University of Korea, College of Medicine (XC15TIMI00240). Informed written consent was
62 obtained from all the enrolled patients. Peripheral whole blood samples of 15 mL each were
63 collected from 22 patients with NSCLC with ALK translocations and from 1 patient with
64 NSCLC patient without an ALK translocation (patient #23 in Table II). The ALK status had
65 previously been confirmed using FISH analysis of biopsied or excised specimens. Of the 15
66 mL of blood sample, 5 mL was used for CTC detection and enumeration using
67 immunofluorescence staining, and the remaining 10 mL was used for CTC detection, culture,
68 and ALK diagnosis using FISH. Additional clinical and pathological information, such as
69 histologic subtype, ALK positivity in biopsied sample, status of metastasis, smoking history,
70 treatment history (chemotherapy or radiotherapy), use of crizotinib, and current disease
71 status, was documented.

72 CTC detection and culture by Cytogen enrichment platform

73 From each patient, 15 mL of blood was collected in acid citrate dextrose tubes and
74 processed within 4 h. Five milliliters of blood was processed through size-based filtration and
75 used for immunofluorescence staining. Briefly, whole blood was separated by density
76 gradient centrifugation, and the peripheral blood mononuclear cells (PBMC) were filtered
77 through a high density microporous (HDM) chip . The retrieved cells from the HDM chip
78 were negatively selected using an immunomagnet (Dynabeads CD45, Thermo Fisher
79 Scientific) to remove remaining white blood cells. Enriched CTCs were placed onto glass
80 slides using Cytospin (Thermo Scientific), fixed in 4% paraformaldehyde for 5 min at room
81 temperature, and kept at 4°C until further processing. The remaining blood samples for CTC
82 culture were processed using a similar procedure, except for the immunomagnetic negative

83 selection.

84 CTC enumeration by immunofluorescence staining

85 Cells were permeabilized with 0.2% Triton-X100 and then quenched with 0.3%
86 hydrogen peroxide. After blocking with 1% BSA in PBS, the cells were incubated with
87 mouse anti-EpCAM antibody (Cell Signaling Technology). EpCAM signals were amplified
88 using a Tyramide Signal Amplification System (goat anti-mouse IgG and Alexa Fluor 488,
89 Life Technologies), which was used according to the manufacturer's instructions. Cells were
90 then incubated with rabbit anti-CD45 antibody (Cell Signaling Technologies) and goat
91 anti-rabbit Alexa Fluor 594 secondary antibody (Invitrogen). The slides were mounted using
92 Fluoroshield with DAPI (ImmunoBioScience). Stained cells were observed and photographed
93 on a Nikon Eclipse Ti fluorescent microscope equipped with a ×400 objective. CTCs were
94 defined as EpCAM positive and CD45 negative cells. For precise identification of CTCs,
95 PC9 cells (EpCAM positive) and KG-1 cells (CD45 positive) were included as positive
96 controls in every immunofluorescence staining.

97 Primary culture of CTCs

98 Enriched CTCs were collected, washed with PBS, and then cultured in 6-well ULA
99 plates (Corning) containing growth medium (human Mesenchymal Stem Cell Growth
100 Medium, Lonza) at 37°C with 5% CO_2. The culture medium was replaced every 3–4 days
101 with minimal disturbance to avoid cell loss. After ~21 days of culture, cell suspensions were
102 fixed in 10% formalin and placed onto glass slides using a liquid-based slide processor
103 (SurePath, Becton Dickinson) according to the manufacturer's instructions.

104 ALK immunocytochemistry

105 Four randomly selected cases that showed a good yield after cultures were assessed
106 using immunostaining for ALK. Immunocytochemical staining was performed using
107 Benchmark Ultra (Ventana, Roche, UK) with a pre-diluted D5F3 clone (Ventana, Roche,
108 UK) according to the manufacturer's instructions.

109 FISH analysis and interpretation

110 EML4-ALK translocation was assessed using Vysis ALK Break Apart FISH probe
111 kit (Abbott Molecular, Des Plaines, IL), which is a US Food and Drug
112 Administration-approved test. It is designed to detect ALK in chromosome 2p23 in
113 formalin-fixed tissue with two adjacent probes: one at the 3′ end (orange) and one at the 5′

114 end (green) of ALK. Pretreatment, protease digestion, overnight probe hybridization,
115 post-washing, and DAPI counter-staining were performed on each sample according to the
116 manufacturer's instructions. For each test, ALK-positive NSCLC tissue was used as a
117 positive control. Normal cells and tumor cells without ALK translocation typically show two
118 fusion signals, while cells with ALK translocation show a characteristic ALK split pattern.
119 Processed slides were automatically scanned using a BioView^{TM} workstation (BioView,
120 Abbott Molecular), and a preliminary interpretation was made by the system.

121 **Results**

122 Patient information

123 Twenty-two patients with NSCLC were included in this study (Table I). All 22 had
124 previously been diagnosed as positive for ALK rearrangement in tumor tissues using Vysis
125 ALK Break Apart FISH Probe Kit. ALK positivity in tissue biopsies was 41.2% (18%–80%).
126 Eight patients were male (36%), and 14 patients were female (64%). The average age was
127 58.4 years (32–82 years), and eight patients (36%) had smoked previously. Among the 22
128 patients, 21 had mucinous carcinomas, and 1 patient had an undifferentiated carcinoma.
129 Twenty patients (81%) had remote metastases. All patients had received chemotherapy or
130 radiation therapy, and 15 patients (68%) had, additionally, been treated with crizotinib. At the
131 time of the study, none of the patients had shown a complete response (0%), the disease was
132 stable in 8 (36%), 3 showed partial responses (14%), and 11 had a progressive disease (50%).

133 CTC detection in patient blood

134 We defined CTCs as EpCAM positive and CD45 negative cells (Figure 1). CTCs
135 were detected in 10 out of 22 patients (45.5%), and the average number of EpCAM positive
136 CTCs was 1.6 (1–8 cells) (Table II). CTC quantification in patient #2 was not possible
137 because of the poor quality of the immunofluorescence staining.

138 Analysis of ALK FISH and ALK immunocytochemistry using cultured CTCs

139 ALK expression in cultured CTCs was analyzed using immunocytochemistry (Figure
140 2) and FISH (Table II). FISH signals were detected in an average of 57.4 cells (range 2–197).
141 ALK rearrangement was observed in 72.7% (16 out of 22 patients), and the average number
142 of positive cells was 4.3 (range 0–13). Representative images of ALK rearrangement
143 demonstrated using FISH are presented in Figure 3.

144 **Discussion**

145 CTCs can be a powerful indicator of cancer diagnosis, metastasis, and prognosis ;
146 therefore, the recent focus has been placed on the clinical relevance of CTC detection and
147 characterization . The characteristics of CTCs are variable because they undergo processes
148 such as epithelial–mesenchymal transition to leave tumor tissue and enter the circulatory
149 system . Additionally, cancer therapies can change the characteristics of CTCs. This
150 phenomenon is one of the major obstacles to CTC-based medical approaches. In the
151 pharmaceutical industry, the importance of anti-cancer drug development and patient-specific
152 medication using CTCs has recently received much attention . However, these studies are
153 very limited due to the changeable characteristics of CTCs under different environmental
154 conditions (*in/ex vitro* vs *in vivo*). Therefore, the accurate characterization of CTCs is
155 essential for their clinical application.

156 In this study, CTCs were isolated from the blood of patients with ALK
157 rearrangement and were cultured to confirm whether the cultured cells maintain the ALK
158 rearrangement. CTCs were identified using the CTC marker EpCAM. In nine patients, CTCs
159 were not identified in the blood, although ALK rearrangement was confirmed after the
160 culture. The CTCs that are epithelial or epithelial–mesenchymal, but not completely
161 mesenchymal, can be detected using EpCAM staining . Therefore, we assumed that the
162 ALK-positive CTCs in these nine cases were mesenchymal.

163 Determining the ALK rearrangement status is crucial for prescribing treatment with
164 ALK kinase inhibitors, such as crizotinib, in patients with NSCLC . FISH,
165 immunohistochemistry, and RT–PCR have been used to detect ALK rearrangements . In this
166 study, we analyzed ALK rearrangements in patients with NSCLC who were already
167 diagnosed as ALK-positive using tissue biopsies. We confirmed ALK rearrangements in
168 CTCs of 16 out of 22 patients.

169 The ALK inhibitor crizotinib has been used to treat ALK-positive patients with
170 NSCLC. Over 60% of such patients respond to crizotinib, and the median progression-free
171 survival is 8–10 months . However, despite the initial response, in the majority of patients,
172 the tumor relapsed because of crizotinib resistance. In recent studies, ceritinib has been found
173 to be an effective treatment option in crizotinib-resistant patients . Therefore, monitoring
174 ALK-positive CTCs during crizotinib treatment using serial liquid biopsies may be a useful
175 tool for making a decision on whether other ALK inhibitors, such as ceritinib, are required.

176 We tested the efficiency of CTC-based ALK rearrangement analysis using FISH and

177 immunocytochemistry. ALK rearrangement was accurately detected using FISH, but the
178 results from immunocytochemistry were unclear in some patients. Based on this finding, we
179 suggest that FISH is a more effective and precise method for detecting ALK rearrangement in
180 CTCs.

181 Analyzing ALK rearrangements in CTCs is a useful method for predicting crizotinib
182 resistance and for choosing additional inhibitor treatment in patients with NSCLC. Our
183 results have demonstrated that detecting ALK rearrangements in CTCs using FISH can be
184 considered as an alternative diagnostic method in patients from whom a tissue biopsy sample
185 cannot be obtained. This procedure is non-invasive and can, therefore, be repeated multiple
186 times to monitor the drug response.

187 In conclusion, we verified the genetic correlations between CTCs and tumor tissue of
188 22 patients with pre-diagnosed ALK rearrangement. We suggest that analysis of ALK
189 rearrangement using FISH in CTCs be considered as an alternative method to tumor tissue
190 biopsies where necessary. In addition, we have uncovered the following important insights
191 into the use of CTCs:

192 1. CTCs represent the genetic profile of primary tumor tissue.

193 2. Culturing CTCs from liquid biopsies is an alternative to tissue biopsies for the detection of
194 genetic variations in patients with cancer.

195 3. In patients with ALK rearrangement, CTCs may be helpful for monitoring the drug
196 response and additional therapy selection.

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199 FISH analysis.

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270
271 **Figure legend**

272 **Figure 1**. Immunofluorescence staining of circulating tumor cells (CTCs) from patients with
273 lung cancer. We identified EpCAM positive and CD45 negative cells as CTCs.

274 **Figure 2.** Immunocytochemical staining of cultured circulating tumor cells (CTCs).
275 Culturing CTCs from each patient blood (A patient #10, B #13, C #21, D #22). Culturing
276 CTCs of patient #10 consisted with high number of ALK positive CTCs, but
277 immunocytochemistry staining intensity is not distinctly distinguished compared with
278 culturing CTCs of patient #13 that is not detected ALK positive CTCs.

279 **Figure 3.** Detection of ALK rearrangements using FISH in culturing CTCs by Vysis ALK
280 Break Apart FISH Probe Kit. ALK positive CTCs from NSCLC patient with ALK
281 arrangement (A #4, B #6, C #7, D #8, E #9, F #14, G #17, H #18, I #19, J #20).

Table I. Clinical characteristics of patients.

	No. (%)	
Gender	Male: 8 (36%)	Female: 14 (64%)
Age	32 ~ 82	Average 58.4
Smoking status	Yes: 8 (36%)	No: 14 (64%)
Histologic subtype	21 Adenocarcinomas (21 Mucinous carcinoma, 1 Undifferentiated carcinoma)	
ALK positive rate in biopsy	18 ~ 80 %	Average 41.2%
Metastasis	Yes: 20 (81%)	No: 2 (9%)
Chemo or Radiation Therapy	Yes: 22 (100%)	No: 0 (0%)
Additional Crizotinib Treatment	Yes: 15 (68%)	No: 7 (32%)
Current Disease status	Complete response: 0 (0%)	Stable disease: 8 (36%)
	Partial response: 3 (14%)	Progressive disease: 11 (50%)

Table II. Detection and enumeration of CTCs from NSCLC patients with ALK arrangement.

Pt.	Gender	Age	EpCAM (+) CTCs	ALK+[a] CTCs	(ALK+* CTCs)/ (Fish+ CTCs) %	Number of CTCs +FISH signals	Crizotinib
#1	F	35	5	3	5%	58	Y
#2	F	82	Failed	0	0%	16	Y
#3	M	59	0	0	0%	3	Y
#4	F	54	2	4	31%	13	N
#5	M	70	7	0	0%	15	Y
#6	M	62	8	6	46%	13	N
#7	F	76	5	13	9%	149	N
#8	M	51	0	4	17%	24	Y
#9	F	49	0	1	50%	2	Y
#10	M	71	1	6	3%	177	Y
#11	M	51	1	3	33%	9	Y
#12	F	32	1	0	0%	53	Y
#13	F	49	1	0	0%	130	Y
#14	F	50	0	4	4%	92	N
#15	F	51	0	0	0%	4	Y
#16	F	63	0	1	1%	88	Y
#17	F	56	0	1	25%	4	Y
#18	M	71	1	3	50%	6	N
#19	F	66	0	3	2%	149	Y
#20	F	53	0	10	62%	16	Y
#21	F	60	0	3	21%	14	N
#22	M	76	0	5	3%	197	N
average			1.6	4.3	7.6%	57.4	
Crizotinib treatment & ALK+ CTCs patients (average)				3.6	6.1%	58.6	
No crizotinib treatment & ALK+				5.4	7.9%	69.1	

CTCs patients (average)							
#23	M	69	1	0	0%	261	N

All patients underwent anti-cancer therapy.

ALK+[a] : Detection of ALK rearrangement

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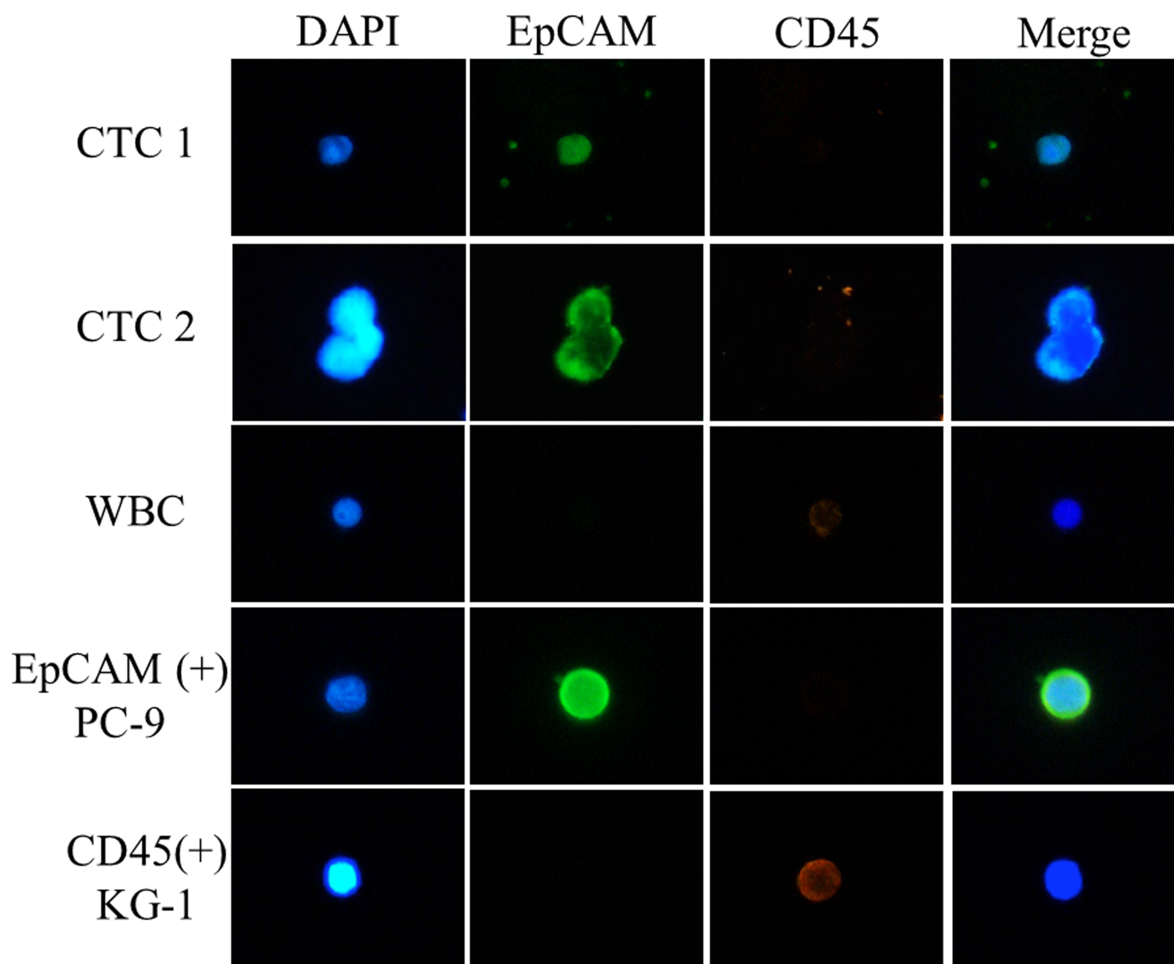

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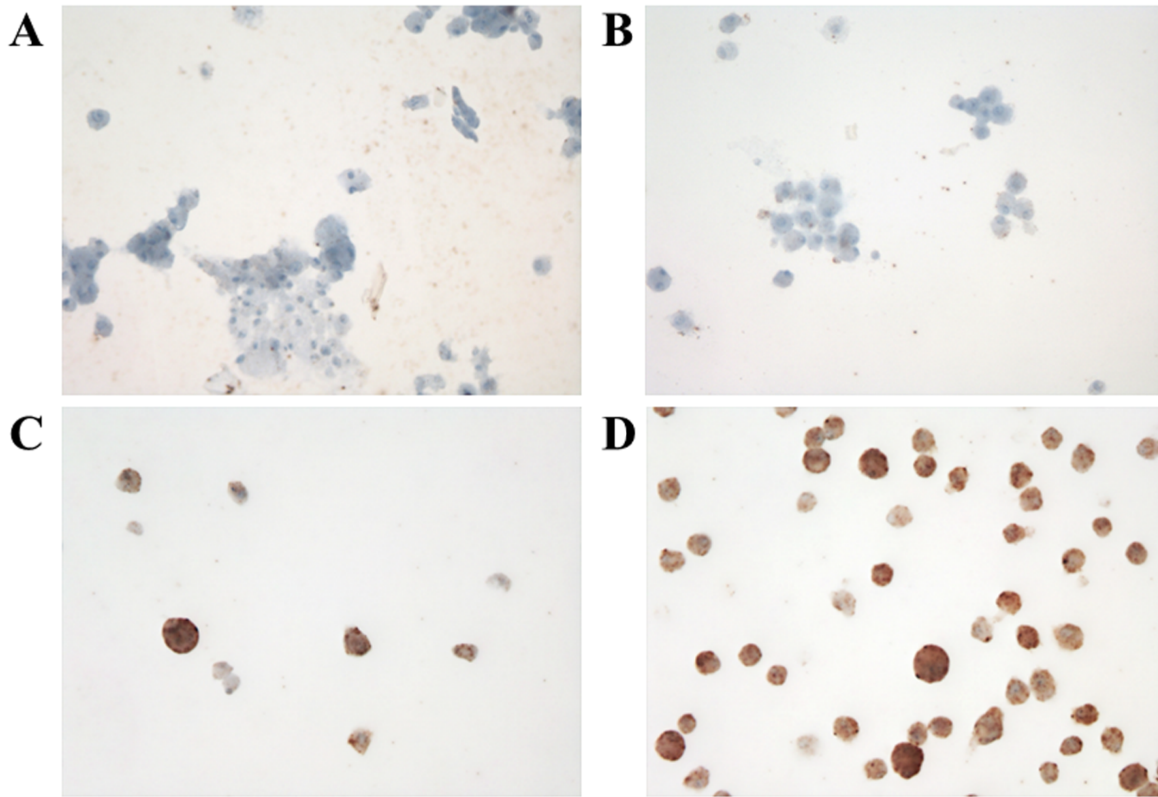

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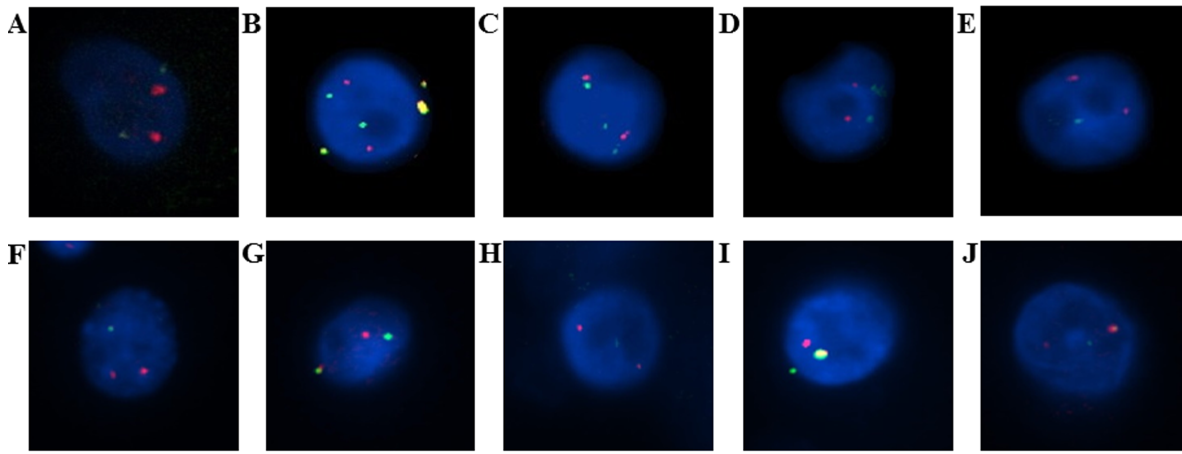

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