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Imaging, Diagnosis, Prognosis

Clinical Cancer Research

Genetically Abnormal Circulating Cells in Lung Cancer Patients: An Antigen-Independent Fluorescence *In situ* Hybridization–Based Case-Control Study

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Abstract

Purpose: We performed a study to determine if a fluorescence *in situ* hybridization (FISH)–based assay using isolated peripheral blood mononuclear cells (PBMCs) with DNA probes targeting specific sites on chromosomes known to have abnormalities in non–small cell lung cancer (NSCLC) cases could detect circulating genetically abnormal cells (CACs).

Experimental Design: We evaluated 59 NSCLC cases with stage I through IV disease and 24 controls. PBMCs and matched tumors were hybridized with 2 two-color [3p22.1/CEP3 and 10q22.3 *(SP-A)*/CEP10) and 2 four-color [CEP3, CEP7, CEP17, and 9p21.3 (URO); and *EGFR*, *c-MYC*, 6p11-q11, and 5p15.2 (LAV)] FISH probes. Percentages of cytogenetically abnormal cells (CACs) in peripheral blood and in matched tumor specimens were quantified by using an automated fluorescent scanner. Numbers of CACs were calculated based on the percentage of CACs (defined as PBMCs with genetic abnormalities) per milliliter of blood and expressed per microliter of blood.

Results: Patients with NSCLC had significantly higher numbers of CACs than controls. Mean number of CACs ranged from 7.23 ± 1.32/μL for deletions of 10q22.3/CEP10 to 45.52 ± 7.49/μL for deletions of 3p22.1/CEP3. Numbers of CACs with deletions of 3p22.1, 10q22.3, and 9p21.3, and gains of URO, increased significantly from early to advanced stage of disease.

Conclusions: We have developed a sensitive and quantitative antigen-independent FISH-based test for detecting CACs in peripheral blood of patients with NSCLC, which showed a significant correlation with the presence of cancer. If this pilot study can be validated in a larger study, CACs may have a role in the management of patients with NSCLC. *Clin Cancer Res; 16(15); 3976–87. ©2010 AACR.*

Cytogenetic evidence exists to show that circulating epithelial cells (CECs) in the bloodstream contain genetic abnormalities similar to those in primary tumors (1). Investigators used a combination of immunohistochemistry and fluorescence *in situ* hybridization (FISH) to show that the aneusomies found in CECs were similar to those detected in primary breast, colon, kidney, and prostate cancers (1). Detection of CECs or circulating tumor cells (CTCs) using a simple blood test may assist in early detection of lung cancer at diagnosis and relapse and provide a minimally invasive way to monitor results of therapy. Researchers have attempted to isolate CTCs from peripheral blood using a variety of techniques, including immunomagnetic capture of EpCAM-positive cells, which were shown to be of clear prognostic use for breast, prostate, and colorectal carcinomas (1–7). However, usually very few CTCs are recovered using these techniques. Other CTC isolation techniques include filtration of CTCs, density gradient separation, microchip technologies, and PCR-based techniques (8–12). There are also limitations to these methods, particularly PCR, which restricts enumeration and morphologic analysis of cells (13, 14).

Relatively few reports have described detection of CTCs in patients with lung cancer. A recent report described positive depletion of $CD45^+$ cells from blood with magnetic beads followed by CK18/19 staining of enriched cells in patients with non–small cell lung cancer (NSCLC; ref. 15). Although few CTCs from these patients

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Translational Relevance

Circulating cytogenetically abnormal cells (CACs) containing similar genetic abnormalities to the primary tumor are present in patients with non–small cell lung cancer (NSCLC). We developed an antigen-independent quantitative fluorescence *in situ* hybridization–based assay that detected circulating CACs from patients with NSCLC using DNA probes to identify genomic aberrations known to be present in patients with NSCLC. Genetic abnormalities in CACs in baseline blood correlated with genetic abnormalities in resected NSCLC. Numbers of CACs correlated with stage and were significantly different from controls. Different from previous studies using immunomagnetic capture for epithelial antigens, with low tumor cell recovery, our approach allowed us to detect much higher numbers of circulating CACs, which may be epithelial, mesenchymal, or stem cell in origin. Assays for CACs may be used as adjuncts to the diagnosis of indeterminate lung nodules, as markers of response to therapy and for detection of minimal residual disease.

were recovered, the number of CTCs generally correlated with the stage of disease, with fewer than two CTCs per 7.5 mL of blood detected in controls. However, relying on expression of epithelial markers in CTCs, including lack of sensitivity to antigenic detection by EpCam, may drastically reduce the sensitivity of CTC detection if the epithelial markers are absent. For example, a recent study pointed to the expression of mesenchymal rather than epithelial markers in common types of breast carcinoma, which may have resulted in undercounting of CTCs (9).

The use of a FISH assay without an epithelial capture component to detect genetically abnormal circulating cells is attractive, as it does not rely on antigenic expression and may reveal more CACs than anticipated, if not limited by an antigenic assay for epithelial differentiation. FISH-based tests detect losses and gains of selected chromosomal regions in the nuclei of cells regardless of the lineage of these cells, and can be used to simultaneously evaluate up to four different DNA probes in a single cell. Rapid automated fluorescent scanning of cells and/or analysis of multicolor fluorescent signals for different genes on a per-cell basis using FISH have enabled the classification and quantitation of cytogenetically abnormal cell (CAC) populations in urine, blood, and sputum (16–19). Based on a previous study of lung adenocarcinomas and squamous cell carcinomas using comparative genomic hybridization (CGH) arrays, we selected two genes that are frequently deleted in NSCLC, 3p22.1 (*GC20*) and 10q22.3 (surfactant protein A1 and A2, *SP-A*), and verified that they were deleted in NSCLC specimens by FISH (18). We previously showed more frequent deletions of these genes in sputum specimens obtained from patients with NSCLC than in controls using FISH (18).

Based on these previous results, we sought to establish in this study whether CACs can be detected in the bloodstream of patients with NSCLC. We hypothesized that the CACs encountered in the circulation would bear similar genetic abnormalities to those detected in NSCLC. We used four sets of genome-specific FISH probes, including 3p22.1/CEP3, 10q22.3/CEP10, and two commercially

Table 1. Demographic characteristics of the study population

Characteristic	Controls (*n* = 24)	Cases (*n* = 59)	Clinical stage				
			IA (*n* = 16)	IB (*n* = 8)	II (*n* = 7)	III (*n* = 10)	IV (*n* = 18)
Gender, *n* (%) male	9 (37.5)	30 (50.8)	11 (78.6)	4 (50.0)	2 (22.2)	5 (50.0)	8 (44.4)
*P**		0.27					
Age (mean ± SEM)	55.5 ± 2.86	66.8 ± 1.36	67.4 ± 2.55	70.63 ± 3.67	61.0 ± 3.01	67.7 ± 2.94	66.9 ± 2.88
*P**		0					
Ever smokers (%)	45.83	89.83	81.25	75	100	90	100
*P**		<0.001					
Pack-years (mean ± SEM)	53.1 ± 12.48	45.0 ± 3.37	44.6 ± 8.41	48.0 ± 6.99	45.4 ± 10.1	45.1 ± 5.97	43.8 ± 6.43
*P**		0.7					
Histology, *n* (%)							
Squamous		12 (20.3)	6 (37.5)	2 (25.0)	2 (28.6)	1 (10.0)	1 (5.6)
Adenocarcinoma		32 (54.2)	9 (64.3)	5 (62.5)	5 (71.4)	4 (40.0)	9 (50.0)
NSC		15 (25.4)	1 (7.1)	1 (12.5)	0 (0.0)	5 (50.0)	8 (44.4)
Patients who died, *n* (%)		22 (37.3)	1 (4.5)	0 (0.0)	0 (0.0)	6 (27.3)	15 (68.2)

NOTE: χ^2 test for association (categorical variables); all *P* values are two-sided and compare controls to all cases.
Abbreviation: NSC, non–small cell carcinoma.
**P* value derived from Mann-Whitney test (continuous variables).

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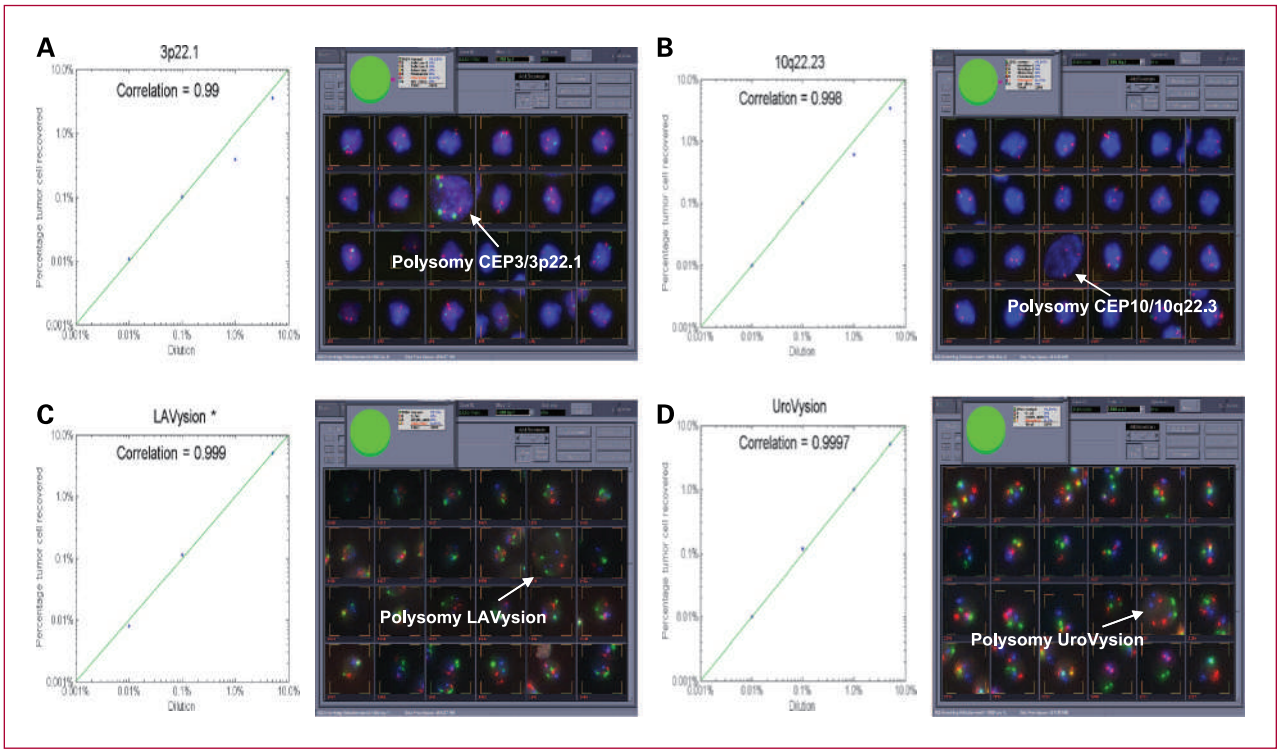

Fig. 1. Calibration curves of actual versus expected recovery at different dilutions of spiked tumor cells showing polysomy (arrows) in a background of normal PBMCs, (A) 3p22.1/CEP3, (B) 10q22.3/CEP10, (C) LAV and (D) URO probes. *Y* axis, percentage of tumor cells recovered; *X* axis, cell dilutions from 0.05 to 0.0001 (*X* and *Y* axes, logarithmic scale).

available probe sets that were selected based on genetic profiles of genes known to be important in the pathogenesis of lung and bladder cancer (20–36) to test peripheral blood mononuclear cells (PBMC) obtained from healthy controls without lung cancer (smokers and nonsmokers) and from patients with NSCLC at different stages. We also tested resected tissue specimens from corresponding primary lung tumors using the same panel of biomarkers.

The main objective of this study was to elucidate and compare the number of CACs between patients with NSCLC and controls, and to determine whether these cells, based on their genetic abnormalities, were indicative of metastasis from the primary tumor. Secondary aims included correlating the presence of the number of CACs in the blood with relapse rate and survival duration, comparing the genetic abnormalities in CACs in the blood with those in the corresponding lung tumors, and developing a quantitative assay for CACs per microliter of blood based on the percentages of CACs in peripheral blood.

Materials and Methods

Study population

In 2007 and 2008, peripheral blood specimens were collected prospectively from 59 patients with NSCLC and 24 controls, including heavy smokers at high risk for lung cancer at The University of Texas M.D. Anderson Cancer Center under an institutional review board–approved protocol. High-risk controls were patients enrolled in a lung cancer screening trial. Cases and controls were not matched by age, smoking status, or sex as seen in Table 1. However, we adjusted for these variables in the analysis.

Demographic characteristics of the study population

Criteria for study entry included no treatment before surgery for stage I-III NSCLC cases. Equal stratification of patients across all NSCLC stages was attempted. Corresponding primary lung tumor tissue specimens were available for 21 patients. The mean ages of controls and patients were 55.5 ± 2.86 and 66.8 ± 1.36 years, respectively (Table 1). Disease stages ranged from early (14 IA, 8 IB, and 9 II) to advanced (10 III and 18 IV), and adenocarcinoma was the most common subtype. Of 23 patients who had a relapse or persistent disease, 4 had an early relapse (within 6 months to 1 year after first treatment). At the time of data analysis, 22 patients had died, most of whom had stage III or IV disease.

Specimen collection

Twenty-five milliliters of blood in EDTA were collected from controls, patients with operable disease immediately before surgery, and patients with inoperable disease at the time of endoscopic ultrasound bronchoscopic evaluation done for staging. Following collection, blood was

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immediately processed by Ficoll-Hypaque density gradient separation. PBMCs were isolated and counted using a Coulter counter (Beckman Coulter), and cytospin preparations of PBMCs containing an average of 10,000 cells were prepared, spray-fixed with alcohol, and stored at −20°C until needed. PBMCs from patients and controls were tested with the same panel of biomarkers. Tumor tissue was available on 21 patients who were enrolled in a lung cancer Specialized Program of Research Excellence study. FISH was performed on both the peripheral blood and tumor tissue to detect concordance of genetic abnormalities in PBMCs and corresponding lung cancer.

Table 2. Distribution of biomarkers in cytogenetically abnormal cells among cases and controls

	Biomarker Percentage (Mean ± SE)		
Marker	**Controls (*n* = 24)**	**Cases (*n* = 59)**	***P***
Deletion 3p22.1/CEP3	2.25 ± 0.40	5.33 ± 0.46	<0.001
Gains 3p22.1/CEP3	0.17 ± 0.07	0.79 ± 0.11	<0.001
Mono 3p22.1/CEP3	0.36 ± 0.13	0.66 ± 0.09	0.02
Poly 3p22.1/CEP3	0.28 ± 0.12	1.18 ± 0.17	<0.001
All abnormalities 3p22.1/CEP3	3.05 ± 0.46	7.96 ± 4.37	<0.001
Deletion 10q22.3/CEP10	0.76 ± 0.21	3.52 ± 0.42	<0.001
Gains 10q22.3/CEP10	0.43 ± 0.11	1.20 ± 0.18	0
Mono 10q22.3CEP10	0.47 ± 0.16	1.11 ± 0.14	0.01
Poly 10q22.3/CEP10	0.01 ± 0.01	0.44 ± 0.07	<0.001
All abnormalities 10q22.3/CEP10	1.67 ± 0.24	6.27 ± 0.53	<0.001
Sum of 3p22.1 and 10q22.3 Deletions	3.01 ± 0.52	8.83 ± 0.72	<0.001
LAVysion deletions			
Single	4.41 ± 0.63	7.31 ± 0.51	0
EGFR	0.77 ± 0.12	1.54 ± 0.18	0.01
5p15.2	0.45 ± 0.17	1.85 ± 0.27	<0.001
c-MYC	0.15 ± 0.06	0.64 ± 0.10	0
6p11-q11	2.99 ± 0.66	3.41 ± 0.35	0.26
LAVysion gains			
Single	2.66 ± 0.37	5.85 ± 0.50	<0.001
EGFR	1.60 ± 0.26	3.86 ± 0.39	<0.001
5p15.2	0.35 ± 0.12	0.68 ± 0.12	0.1
c-MYC	0.14 ± 0.08	0.22 ± 0.06	0.25
6p11-q11	0.65 ± 0.16	1.02 ± 0.14	0.18
LAVysion abnormal cells	0.65 ± 0.22	2.18 ± 0.26	<0.001
UroVysion deletions			
Single	5.36 ± 0.75	8.31 ± 0.43	<0.001
CEP3	0.20 ± 0.06	0.21 ± 0.06	0.55
CEP7	0.15 ± 0.06	1.06 ± 0.18	<0.001
9p21.3	0.89 ± 0.21	1.49 ± 0.23	0.09
CEP17	4.17 ± 0.33	5.54 ± 0.33	0.01
UroVysion gains			
Single	2.23 ± 0.31	5.35 ± 0.42	<0.001
CEP3	1.22 ± 0.19	3.26 ± 0.32	<0.001
CEP7	0.52 ± 0.14	0.73 ± 0.13	0.47
9p21.3	0.23 ± 0.10	0.64 ± 0.10	0
CEP17	0.26 ± 0.07	0.64 ± 0.10	0.05
UroVysion abnormal cells	0.77 ± 0.13	2.11 ± 0.19	<0.001
Sum of UroVysion and LAVysion abnormal cells	1.42 ± 0.291	4.29 ± 0.37	<0.001

NOTE: *P* value was derived from Mann-Whitney test; all *P* values are two-sided per microliter. Enumeration of FISH signals: 3p22.1 (green)/CEP3 (red) or 10q22.3 (green)/CEP10 (red)—normal: 2 signals for each probe; deletion: loss of one or both signals of 3p22.1 or 10q22.3 (green); monosomy: loss of signal for both probes (one red and one green signal); polysomy: more than two signals of each probe (three red and three green or more); gain: gain of one or both signals of 3p22.1 or 10q22.3 (green). LAVysion or UroVysion probes—normal: two signals for each probe, loss: monosomy (one signal) of one probe; gain: polysomy (more than two signals) of one probe; abnormal cells: abnormality of two probes in one cell, loss or gain.

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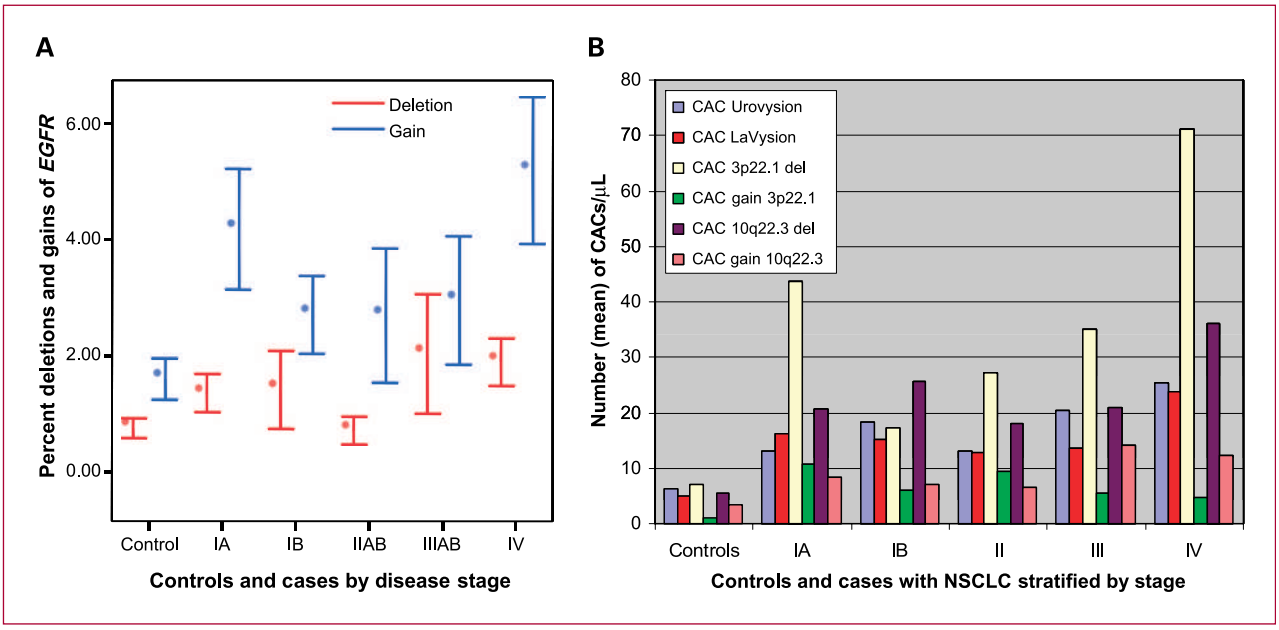

Fig. 2. A, error bar plots showing percentage deletions and gains of *EGFR* (*Y* axis) with the LAV probe set in PBMC specimens obtained from controls and cases with NSCLC by disease stage (*X* axis). B, mean CACs/μL in controls and cases with NSCLC with chromosomal abnormalities of 3p22.1/CEP3, 10q22.3/CEP10, and UroVysion and LAVysion probes stratified by stage. Note the trend for numbers of CACs for most chromosomal abnormalities to increase from early to advanced stage of NSCLC.

FISH testing for cytogenetically abnormal PBMCs (CACs)

The following panel of FISH probes was used: (*a*) a combination of two probe sets: locus-specific identifier (LSI) 3p22.1 with corresponding centromeric probe CEP3 and LSI 10q22.3 (*SP-A*) with corresponding CEP10 prepared in-house as described previously (18, 21–23) and (*b*) two commercially available probe sets containing four probes each—LAVysion (LAV): *EGFR*, *c-MYC*, 6p11-q11, and 5p15.2; and UroVysion (URO): CEP3, CEP7, CEP17, and $p16^{INK4A}$ 9p21.3 (Abbott Molecular). Fluorescent signals in specimens were quantitated on a per-cell basis using an automated fluorescent system (Bioview) that is capable of scanning and classifying hundreds of cells under fluorescent illumination and allows for detection of rare cells according to FISH pattern (19). Using two-color FISH with 3p22.1/CEP3 and 10q22.3/CEP10, a mean of 250 PBMCs was accumulated for each probe set and reviewed for appropriate morphology (round or oval cells) and to verify the number of FISH signals displayed by the program on a per-cell basis by an experienced observer blinded to the disease status. Similarly, at least 200 PBMCs were selected and scored for genomic abnormalities using both URO and LAV four-color probe sets. Cytogenetic abnormalities were scored based on the presence of chromosomal deletions, gains, monosomy, polysomy, or the sum of all abnormalities combined and expressed as percentages of CACs (for detailed methods of scoring biomarker abnormalities, see Supplementary Material).

CAC quantitation

The number of CACs per microliter of blood was calculated as the percentage of CACs (for a specific chromosomal probe set) × the total number of PBMCs isolated/mL of blood collected/1,000. Thus, the number of CACs with deletions or gains of 3p22.1 compared with CEP3 and the number of CACs with deletions or gains of 10q22.3 compared with CEP10 per microliter were calculated. CACs per microliter were calculated for the URO and LAV probe sets based on the presence of at least two chromosomal abnormalities in the biomarkers tested in each nucleus.

Tumor wash specimens

Cell suspensions of tumors were available from 21 patients with resectable tumors, and cytospins of tumor cells were prepared. FISH was done for the 3p22.1/CEP3, 10q22.3/CEP10, the URO and LAV probe sets and evaluated as detailed in Supplementary Materials. A subset of tumors was further classified according to clonal heterogeneity based on chromosomal abnormalities for the different DNA biomarkers (Supplementary Table S6).

Recovery of lung adenocarcinoma cell line experiments

The sensitivity of the FISH-based assay to detect the presence of CACs in peripheral blood was evaluated by performing recovery experiments in which H1299 lung adenocarcinoma cells (AC) were spiked into PBMCs isolated from healthy donors. Four separate dilution assays at

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1 AC/10,000, 1 AC/1000, 1 AC/100, and 5 AC/100 were done. The spiked cell mixtures were hybridized with 3p22.1/CEP3, 10q22.3/CEP10, the LAV set, and the URO set. H1299 cells and PBMC controls were similarly hybridized and evaluated for cytogenetic abnormalities. Calibration curves depicting the number of cells recovered for each biomarker at the different serial dilutions are shown in Fig. 1 and Supplementary Table S1.

Statistical analysis

Descriptive statistical analyses, including the Spearman χ^2 test, were used to test for distributional differences between patients and controls according to categorical variables, and the Mann-Whitney test was used to determine differences in continuous variables. The Mann-Whitney test was also used to test for differences in each biomarker between the patients and controls. Simple linear regression analysis was done to test for trends in the biomarkers by disease stage. Two-sided *P* values were used to determine the level of significance for each test.

To evaluate the role of each biomarker in cancer recurrence and overall survival, we dichotomized each variable into two groups based on the 75th percentile of the controls for each respective outcome. Time to recurrence was defined as the number of months from the date of first treatment to that of first recurrence. Overall survival time was defined as the number of months from the date of first treatment to that of death. Patients lost to follow-up or those patients who had no recurrences or did not die before the end of the study were censored. The Kaplan-Meier method was used to identify any significant differences in time to recurrence and overall survival between the high and low groups for each biomarker, respectively. The time to recurrence and overall survival between the high and low biomarker groups was compared using the log-rank test. Biomarkers found to be significant at the 10% level in the log-rank test were further evaluated using the Cox proportional hazards model adjusted for age, sex, and disease stage.

Results

Cell recovery validation study

The rate of identification of H1299 carcinoma cells based on cytogenetic abnormalities exceeded 99%, whereas unspiked PBMCs with >1% chromosomal abnormalities were not identified. Tumor cell recovery rates for H1299 using polysomy of 3p22.1/CEP3 and 10q22.3/CEP10 DNA probes to quantitate tumor cells at 0.01%, 0.1%, 1.0%, and 5.0% dilution levels ranged from 0.011%, 0.102%, 0.4%, and 3.6%, respectively, for the 3p22.1/CEP3 probe set, and similar recovery rates were noted for the 10q22.3/CEP10 (Fig. 1; Supplementary Table S1). For URO and LAV probe sets, tumor cells were counted only if two abnormal chromosomes per nucleus were present and actual recovery of H1299 carcinoma at 0.01% to 5.0% dilutions ranged from 0.008% to 5.0% for the LAV set and 0.01% to 5.0% for the URO probe set (Fig. 1; Supplementary Table S1). These results showed that the assay recovered the correct proportion of abnormal cells throughout the range of the dilutions from 1/20 (5%) down to 1/10,000 (0.01%).

CACs by case, control status, and NSCLC stage

We recorded the mean percentage of circulating CACs in cases and controls and stratified them by pathologic stage or clinical stage in cases that were inoperable because of advanced disease (IIIB or IV). There was a significant trend for percentages of CACs for all biomarkers, except for the LAV probe set, to increase from early-stage to advanced-stage disease. We noted highly significant differences in the biomarker distribution between patients and controls (Table 2). For example, the mean ± SEM percentage of CACs in the controls ranged from 0.17 ± 0.07 for 3p22.1/CEP3 gains to 3.05 ± 0.46 for combined 3p22.1/CEP3 chromosomal abnormalities. In comparison, the mean ± SEM percentage of CACs for patients with stage IA NSCLC ranged from 1.11 ± 0.30 for 3p22.1/CEP3 gains to 7.00 ± 0.93 for combined 3p22.1/CEP3 abnormalities. Both *EGFR* deletions and gains were significantly different between cases and controls (Fig. 2A; Table 2).

The mean numbers ± SEM of CACs per microliter (derived from the percentages of CACs) are depicted according to the stage of disease, and cases were significantly different compared with controls for all biomarker abnormalities recorded (Fig. 2B; Table 3). Expressed per milliliter, the mean number of CACs for all cases of NSCLC ranged from 7,230 ± 1,320 for gains of 3p22.1/CEP3 to 45,520 ± 7,490 for deletions of 3p22.1/CEP3, whereas for URO and LAV abnormalities, mean CACs were 18,790 ± 3,160 and 17,570 ± 2,820, respectively.

Biomarker abnormalities associated with tumor stage

Many CACs were significantly associated with early stage IA and/or advanced stage (IIIA, IIIB, or IV) NSCLC ($P < 0.05$; Supplemental Table S3). Most notable were CACs containing abnormalities of 3p22.1/CEP3 and 10q22.3/CEP10, and gain or loss of biomarkers in the URO set, which increased significantly from early- to advanced-stage disease (Supplementary Fig. S1).

Correlation between blood and corresponding lung cancer tissue

We obtained paired sets of peripheral blood and tumor tissue from 21 patients who underwent surgical resection of their lung tumors. The same set of FISH probes was used in both PBMCs and tumor specimens. We observed a strong overall correlation between eight biomarker abnormalities in PBMCs and corresponding biomarkers in the tumor washes; specifically, six were positively correlated and included gains of *EGFR*, *c-MYC*, 6p11-q11, 3p22.1, and different abnormalities in the URO set (Supplementary Table S5). Overall, *EGFR* gain in CACs was significantly correlated with *EGFR* gains in tumor washes, especially among patients who presented with stage III and IV disease ($P \leq 0.01$). We observed positively correlated chromosomal

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Table 3. Cytogenetically abnormal cells by biomarker stratified by pathologic stage of NSCLC expressed per microliter of peripheral blood: cases versus controls

		CACs (mean ± SEM)		
	Controls (mean ± SEM), *n* = 24	IA (mean ± SEM), *n* = 16	IB (mean ± SEM), *n* = 8	II (mean ± SEM), *n* = 7
CAC/μL peripheral blood				
UroVysion	6.31 ± 1.19	13.04 ± 3.30	18.37 ± 5.39	13.10 ± 5.86
LAVysion	4.99 ± 1.37	16.19 ± 3.43	15.10 ± 3.76	12.88 ± 7.50
3p22.1 del/CEP3	7.04 ± 2.81	43.75 ± 10.58	17.28 ± 6.87	27.09 ± 11.95
3p22.1 gain/CEP3	1.12 ± 0.50	10.65 ± 3.11	6.08 ± 2.84	9.30 ± 5.09
10q22.3/CEP10 deletions	5.58 ± 1.44	20.72 ± 16.25	25.74 ± 7.90	18.15 ± 6.87
10q22.3 gain/CEP10	3.44 ± 0.95	8.46 ± 1.70	7.08 ± 3.19	6.52 ± 2.33

(*Continued on the following page*)

abnormalities in the CACs and those in the tumor cells by the URO probe set. In contrast, the genetic abnormalities in the LAV set in CACs were negatively correlated with those in the tumor washes. An example of CACs and corresponding tumor cells for all biomarkers quantitated is depicted in Fig. 3A and B.

Biomarker abnormalities associated with disease recurrence

Twenty-three (39%) patients had disease recurrence. The median time to recurrence was 29 months (95% confidence interval, 15.49–42.51 months). Twenty biomarkers were significant at the 10% level, of which 12 were significant at the 5% level in Kaplan-Meier analyses for disease recurrence (Supplementary Fig. S2; Table 4). Of these, three were significant at the univariate level using the Cox proportional hazards model, namely 5p15.2 gain, 3p22.1 deletion, and a single URO gain. However, these biomarkers were not significant for disease recurrence after adjustment for age, sex, and disease stage.

Biomarker abnormalities associated with overall duration of survival

Twenty-two (37%) of the patients died over a period of less than 1 month to 39 months following collection of baseline blood samples. The median overall survival duration was 29 months (95% confidence interval, 12.69–45.31 months). Six biomarkers were significant at the 10% level in Kaplan-Meier analyses for overall survival (Supplementary Fig. S3), but only two were significant at the univariate level: *EGFR* deletions and a single URO gain. However, these biomarkers were not significant for overall survival after adjustment for age, sex, and disease stage (Table 4).

Discussion

To our knowledge, this is the first study to use a quantitative FISH-based assay to detect cytogenetically abnormal circulating cells from patients with stage (I–IV) NSCLC and controls, using specific probes to identify previously characterized genomic aberrations described in NSCLC. We hypothesized that CACs encountered in the circulation would bear similar genetic abnormalities to those detected in NSCLC. However, to prove that these CACs are of epithelial origin, current standards require demonstration of expression of epithelial markers such as cytokeratin or EpCAM, with negative expression of CD45, a hematolymphoid marker. Alternatively if CACs derived from NSCLCs have undergone an epithelial to mesenchymal transition, antigenic expression of vimentin, SNAIL, TWIST1, or other markers of epithelial to mesenchymal transition with concurrent demonstration of genetic abnormalities by FISH would be required (37). If circulating cancer stem cells with cytogenetic abnormalities are present, demonstration of ALDH1 or CD133 in these cells would be needed (38).

In addition, it is possible that other cells present in the circulation, including endothelial precursor cells, lymphocytes, monocytes, and hematopoietic stem cells, may express aberrant genetic markers. Cytogenetic abnormalities have previously been shown in peripheral blood lymphocytes of patients with lung cancer that were identical with those of the primary tumor (39).

Previous investigators used FISH to detect aneuploidy in CTCs obtained by immunomagnetic capture for epithelial antigen followed by immunohistochemistry for cytokeratin or prostate-specific antigen (40, 41). Although these studies showed genetic abnormalities similar to those of the primary tumor, they were limited by a low cell recovery and inability to detect chromosomal abnormalities in patients with <10 CTCs per 7.5 mL blood (17, 40, 41). To detect CACs, we used an automated fluorescence-based scanner to analyze a minimum of 900 PBMCs obtained by density gradient enrichment and hybridized for 12 different DNA biomarkers in all patients and controls. Although we hesitate to call these CACs "circulating epithelial cells" based on lack of phenotypic evidence for epithelial cell differentiation, we assume that the CACs

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Table 3. Cytogenetically abnormal cells by biomarker stratified by pathologic stage of NSCLC expressed per microliter of peripheral blood: cases versus controls (Cont'd)

	CACs (mean ± SEM)				
	III (mean ± SEM), *n* = 10	IV (mean ± SEM), *n* = 18	Cases (mean ± SEM), *n* = 59	MW*	Trend†
CAC/μL peripheral blood					
UroVysion	20.44 ± 7.00	25.37 ± 8.58	18.79 ± 3.16	0.01	0.14
LAVysion	13.49 ± 3.03	23.89 ± 7.82	17.57 ± 2.82	<0.001	0.35
3p22.1 del/CEP3	34.94 ± 10.35	71.12 ± 19.82	45.52 ± 7.49	0.01	0.13
3p22.1 gain/CEP3	5.56 ± 1.61	4.76 ± 2.13	7.23 ± 1.32	<0.001	0.09
10q22.3/CEP10 deletions	20.86 ± 4.93	35.98 ± 8.52	25.78 ± 3.28	<0.001	0.12
10q22.3 gain/CEP10	14.22 ± 7.92	12.38 ± 5.06	10.21 ± 2.12	0.01	0.33

NOTE: All *P* values are two-sided (to express number per milliliter, multiply by 1,000). Enumeration of FISH signals: 3p22.1 (green)/CEP3 (red) or 10q22.3 (green)/CEP10 (red)—normal: two signals for each probe; deletion: loss of one or both signals of 3p22.1 or 10q22.3 (green); monosomy: loss of signal for both probes (one red and one green signal); polysomy: more than two signals of each probe (three red and three green or more); gain: gain of one or both signals of 3p22.1 or 10q22.3 (green). LAVysion or UroVysion probes—normal: 2 signals for each probe, loss: monosomy (one signal) of one probe; gain: polysomy (more than two signals) of one probe; abnormal: abnormality of two probes in one cell, loss or gain.

**P* value derived from Mann-Whitney test (all cases versus controls).

†*P* value for trend of abnormality across tumor stage.

detected may in fact be CTCs derived from NSCLC based on the following findings: (*a*) eight of the DNA biomarkers in the PBMCs were significantly correlated with the resected lung tumors; (*b*) the mean percentages of genetically altered cells for all 12 biomarkers tested correlated with the stage of NSCLC, with the lowest levels detected in patients with stage I disease and the highest detected in patients with stage III and IV disease; (*c*) the controls had significantly fewer genetically abnormal cells for all the biomarkers; (*d*) at the 0.0001 dilution level of spiked tumor cells in PBMCs from buffy coats of normal healthy donors, genetic abnormalities, such as polysomy for 10q22.3/CEP10 or 3p22.1/CEP3 genes, or abnormal cells by FISH for either of two chromosomal abnormalities in the multiprobe biomarker sets, were not shown in the PBMCs.

Detection of CACs in control subjects may be due to the fact that some were heavy smokers and exposed to the same tobacco carcinogens as the cancer patients. The presence of chromosome damage in healthy controls at significantly lower levels than in cancer patients has been reported (42, 43). Moreover, there is evidence that abnormalities in cytogenetic biomarkers are positively correlated with cancer risk, reflecting the genotoxic effects of carcinogens as well as individual susceptibility to cancer development (44–46).

In comparison with other methods that used EpCAM-positive cells to detect CTCs, we observed several orders of magnitude higher numbers of CACs in our antigen-independent genetic assays than most other studies. Depending on the biomarker abnormality assayed, up to 45 CACs per microliter were detected compared with <10 CACs per 1 milliliter in most studies using immunomagnetic beads (2, 6, 15, 17, 41). The percentages of CACs tended to increase with disease stage, reflecting tumor burden. Certain genetic abnormalities were associated with relapse and overall survival duration; however, after adjustment for age, sex, and disease stage, these were not statistically significant, most likely reflecting a relatively small number of patients and the strong effect of disease stage on overall survival.

CACs with certain chromosomal abnormalities were present in the blood of patients with both early- and advanced-stage NSCLC and were correlated with relapse and poor survival, suggesting that genetic abnormalities that persist throughout all stages and at relapse may represent cancer stem cell markers.

Although the primary lung tumors manifested a wide range of clonal heterogeneity, including subpopulations with high chromosomal copy number, the usual phenotype for CACs in blood was a single loss or gain of a gene or chromosome, which correlated with the presence of similar clones in the tumor (Supplementary Table S6). Our study is the first to show that high levels of CACs are present in the bloodstream of patients with NSCLC according to detection of genetic abnormalities using antigen-independent FISH-based assays. Our ongoing research shows that although most CACs are negative for CD45, a hematolymphoid marker, a low percentage of CACs are cytokeratin positive and may coexpress CD45. Future goals include studies with larger numbers of patients in both early and advanced disease to confirm that CACS are related to disease stage, relapse, and survival, as well as evaluation of epithelial,

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mesenchymal, stem cell, and hematolymphoid markers, combined with FISH, to discover the origin and phenotype of the CACs. If, as we suspect, CACs actually represent CTCs, then assays for CACs may be used for clinical applications. These would include use of CACs as an adjunct to the diagnosis of radiographically detected indeterminate lung nodules, or as markers of response to cancer therapy and for detection of minimal residual disease.

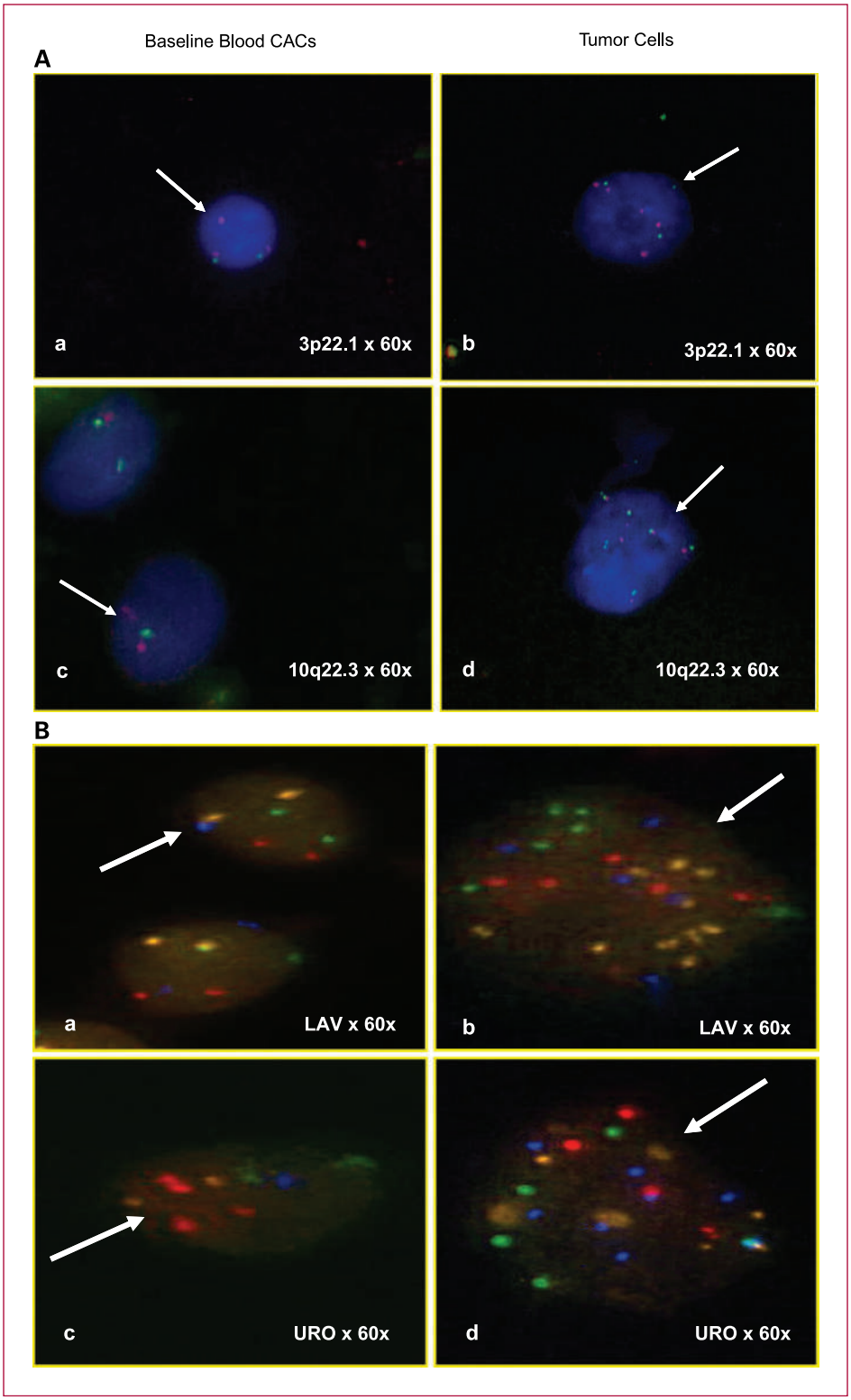

Fig. 3. FISH of baseline blood and corresponding tumor showing correlating genetic abnormalities from a patient with stage IA adenocarcinoma: A, a and b, deletions 3p22.1; CEP3 (red), 3P22.1 (green). c, deletions 10q22.3; d, polysomy 10q22.3/CEP10; CEP10 (red), 10q22.3 (green). B, a, monosomy 6p11-q12; b, amplification *EGFR*, *c-MYC*; *EGFR* (red) 5.p15.3 (green), 6p11-q12 (aqua), *c-MYC* (yellow). c, trisomy CEP3 and monosomy CEP17; d, polysomy CEP3, CEP7, CEP17, and 9p21.3. CEP3 (red), CEP7 (green), CEP17 (aqua), 9p21.3 (yellow).

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Table 4. Markers associated with disease recurrence and survival

Markers	Median survival (mo), 95% CI		*P**	Cox model unadjusted HR (95% CI)	Cox model adjusted HR† (95% CI)
	Low	High			
Recurrence					
3p22.1/CEP3					
Deletions	—	18.8 (14.2–23.5)	0.07	—	—
Monosomy	90.5 (–)	18.9 (10.8–26.9)	0.02	—	—
Combined abnormalities	—	18.9 (9.5–28.2)	0.03	—	—
3p22.1/10q22.3					
Deletions	29.0 (–)	15.5 (9.5–21.5)	0.02	—	—
10q22.3					
Monosomy	90.5 (–)	16.0 (9.7–22.3)	0.07	—	—
Combined abnormalities	29.0 (10.7–47.3)	18.9 (9.9–27.8)	0.02	—	—
LAVysion					
EGFR deletions	—	18.9 (10.2–27.6)	0.03	—	—
6p deletions	15.5 (12.3–18.7)	90.4 (–)	0.01	—	—
Single gain	90.4 (–)	18.7 (14.0–23.7)	0.07	—	—
5p gain	29.0 (10.0–48.0)	16.0 (9.5–22.5)	0.05	3.04 (1.15–8.01)	1.57 (0.55–4.51)
6p gain	19.2 (7.6–30.8)	16.0 (8.9–23.1)	0.04	—	—
Abnormal	29.0 (14.8–43.2)	18.9 (10.9–26.8)	0.09	—	—
UroVysion					
CEP3 Deletions	38.4 (–)	16.0 (0.0–34.1)	0.07	4.03 (1.39–11.64)	1.87 (0.64–5.51)
9p21.3 Deletions	37.4 (0.0–80.8)	15.5 (12.4–18.6)	0	—	—
Single gain	38.4 (4.2–72.6)	16.0 (10.1–22.0)	0	3.24 (1.13–9.26)	2.14 (0.67–6.79)
CEP3 gain	38.4 (19.9–56.8)	16.0 (11.7–20.4)	0	—	—
Abnormal	29.0 (11.5–46.5)	18.9 (9.8–28.0)	0.03	—	—
CACs/μL Peripheral Blood					
UroVysion	38.4 (10.6–66.1)	18.9 (7.8–30.0)	0.06	—	—
UroVysion/LAVysion	29.0 (11.0–47.0)	18.9 (10.3–27.4)	0.05	—	—
3p22.1/CEP3 deletions	—	19.2 (7.1–31.3)	0.1	—	—
Survival					
10q22.3/CEP10 Combined abnormalities	29.0 (9.9–48.1)	19.2 (10.3–28.0)	0.07	—	—
3p22.1 and 10q22.3 Deletions	29.0 (–)	18.9 (14.2–23.5)	0.06	2.55 (1.02–6.38)	1.32 (0.47–3.72)
EGFR deletions	—	19.2 (9.5–28.9)	0.05	—	—
9p21.3 deletions	28.4 (6.0–70.7)	19.2 (14.9–23.4)	0.05	—	—
UroVysion Single gain	38.4 (6.2–70.6)	18.9 (14.4–23.4)	0.02	3.62 (1.38–9.50)	2.51 (0.89–7.06)
CEP3 gain	29.0 (10.0–48.0)	18.9 (15.2–22.6)	0.03	—	—

NOTE: Enumeration of FISH signals: 3p22.1 (green)/CEP3 (red) or 10q22.3 (green)/CEP10 (red)—normal: two signals for each probe; deletion: loss of one or both signals of 3p22.1 or 10q22.3 (green); monosomy: loss of signal for both probes (one red and one green signal); polysomy: more than two signals of each probe (three red and three green or more); gain: gain of one or both signals of 3p22.1 or 10q22.3 (green). LAVysion or UroVysion probes—normal: two signals for each probe, loss: monosomy (one signal) of one probe; gain: polysomy (more than 2 signals) of one probe; abnormal: abnormality of two probes in one cell, loss or gain.

Abbreviations: HR, hazard ratio; CI, confidence interval.

*P value from Kaplan-Meier log-rank test.

†HR adjusted by age, sex, and stage; —, median survival estimates were not calculable.

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Disclosure of Potential Conflicts of Interest

R.L. Katz: commercial research grant, AstraZeneca; patent pending.

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Clinical Cancer Research

Genetically Abnormal Circulating Cells in Lung Cancer Patients: An Antigen-Independent Fluorescence *In situ* Hybridization–Based Case-Control Study

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