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ORIGINAL PAPER

A potential probe set of fluorescence in situ hybridization for detection of lung cancer in bronchial brushing specimens

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Abstract
Purpose The study aims to find candidate probes of fluorescence in situ hybridization (FISH) for detection of lung cancer with bronchial brushings and to evaluate whether the accuracy of diagnosing lung cancer by cytological deviant and genetic abnormalities is greater than that of cytology alone.
Methods Centromeric enumeration probes (CEPs) for chromosomes 2, 3, 6, 7, 8, 9, 11, 12, and 17 were analyzed using FISH in 74 surgical resection tissues, 32 operative margin tissues without tumor involvement of lung cancer, and 174 bronchial brushings.
Results The aneuploidy rates of the tested probes were 61.7, 89.1, 80.0, 92.7, 65.0, 70.4, 66.7, 71.8, 68.9 % in tumor tissues, and 29.3, 58.9, 33.3, 69.6, 67.0, 40.3, 38.0, 49.3, 35.1 % in bronchial brushings. The combination of cytology and FISH using the three-probe set for chromosomes 3+7+8 significantly improved the sensitivity of bronchial brushing examination for lung cancer detection ($P = 0.00003$), especially squamous cell carcinoma (SCC), which increased from 78.0 to 98.2 %. The specificity of the 3+7+8 probe set was 94.6 %. Moreover, a high aneuploidy rate of the probe set in bronchial brushings was detected more often in SCCs ($P = 0.029$) and late-stage non-small-cell lung cancer (NSCLC) ($P = 0.044$). Kaplan–Meier curves indicated that adenocarcinoma (ADC) patients with high aneuploidy rate of CEP3 in tissue samples exhibited poorer overall survival ($P = 0.016$).
Conclusions FISH performed on cytology preparations is useful for confirmation of cancer diagnosis. The three-probe set, 3+7+8, has potential value for the detection of SCCs in bronchial brushings.

Keywords Lung cancer · Fluorescence in situ hybridization · Probe · Cytology diagnosis

Abbreviations

ADC	Adenocarcinoma
CEP	Centromeric enumeration probes
FISH	Fluorescence in situ hybridization
NSCLC	Non-small-cell lung cancer
OS	Overall survival
SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer

Introduction

Lung cancer is the leading cause of cancer incidence and mortality among men and women throughout the world. In China, during the past 30 years, mortality from lung cancer has increased by 465 % (Wen and Dehnel 2011). Cancer Statistics 2011 reported that only about 16 % of patients with lung cancer can survive over five years, and the

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survival rate of late-stage patients is 4 %, despite progress in surgical techniques and chemotherapy (Siegel et al. 2011). More than two-thirds of cases are diagnosed at a late stage without the option of optimal therapy. However, when diagnosed at stage I, the survival rate could be up to 60–80 % (Agarwal et al. 2010). Therefore, early diagnosis is vitally important for improved prognosis.

Cytology detection in endobronchial brushings is a clinically convenient test for the diagnosis of lung cancer. The sensitivity of this technique for centrally located tumors is 44–94 % (mean 72 %). However, the sensitivity varies from 6 to 83 % (mean 45 %) in peripheral tumors (Mazzone et al. 2002). The use of other methods is important to improve the clinical efficacy of cytology detection.

Lung cancer is characterized by aneuploidy, including the gain and loss of genetic material (Balsara and Testa 2002; Choma et al. 2001; Tonon et al. 2005). Fluorescence in situ hybridization (FISH) is a rapid and sensitive molecular cytogenetic technique that has been used in bronchial brushings for the detection of aneuploidy in lung cancer (Halling et al. 2006; Nakamura et al. 2005; Bubendorf et al. 2005; Yendamuri et al. 2008). However, the optimal application of probes for lung cancer diagnosis is not clear.

In the present study, we examined tumor tissues and bronchial brushings from lung cancer patients using FISH with centromeric probes that were specific for chromosomes 2, 3, 6, 7, 8, 9, 11, 12, and 17, which are frequently aberrant in the disease (Balsara and Testa 2002; Panani and Roussos 2006). We explored the proper probe combinations and compared cytology and FISH detection in bronchial brushings, in order to identify candidate probe set and evaluate the feasibility of applying FISH to complement cytology in detecting lung cancer with bronchial brushings.

Materials and methods

Patients and samples

All specimens were collected in the Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College (CAMS & PUMC) from 2006 to 2008, including 74 surgically resected NSCLC tissues (31 SCCs and 43 ADCs), 32 operative margin tissues without tumor involvement, and 174 bronchial brushing specimens from 59 SCC, 34 ADC, 36 small cell lung cancer (SCLC), and 45 benign lesion patients. All of the samples in this study were residual specimens after diagnostic sampling. None of the patients received treatment prior to surgery or bronchoscopy examination, and all patients signed separate informed consent forms for sampling and research.

Tissue samples were collected shortly after surgery and were stored at -70 °C before use. The brushes were targeted at macroscopically abnormal findings under bronchoscopy. Portions of the brushing specimens were stained using the Papanicolaou method and evaluated by experienced cytopathologists. The residual samples were kept in PreservCyt Solution (Hologic, Inc) until processed. All 174 bronchial brushings were confirmed during a later clinical course in Cancer Hospital, CAMS & PUMC by pathological results from biopsies or surgeries. Basic features of both specimens were recorded concerning the clinical/pathological parameters of tumors (Table 1).

Table 1 Basic clinicopathological data of 74 tissue samples and 129 bronchial brushings from patients with lung cancer

Parameter	No. of tissue samples (%)	No. of bronchial brushings (%)
Age (year)		
Median	59	57
Range	41–80	37–80
Sex		
Male	60 (81.1)	101 (78.3)
Female	14 (18.9)	28 (21.7)
Tumor type		
SCC	31 (41.9)	59 (45.7)
ADC	43 (58.1)	34 (26.4)
SCLC	–	36 (27.9)
NSCLC stage		
I	21 (28.4)	10 (15.1)
II	25 (33.8)	14 (21.2)
III	26 (35.1)	31 (47.0)
IV	2 (2.7)	11 (16.7)
T status		
T1	7 (9.5)	4 (6.3)
T2	47 (63.5)	35 (55.6)
T3	14 (18.9)	13 (20.6)
T4	6 (8.1)	11 (17.5)
N status		
N0	37 (50)	16 (25.4)
N1–3	37 (50)	47 (74.6)
M status		
M0	72 (97.3)	56 (84.8)
M1	2 (2.7)	10 (15.2)
Grade		
G1 + G2	35 (47.3)	24 (39.3)
G3	39 (52.7)	37 (60.7)
Gross pathology		
Central type	36 (48.6)	95 (73.6)
Peripheral type	38 (51.4)	34 (26.4)

In bronchial brushings, the tumor stage and grade were based on pathological results from patients who had surgery. Missing or unknown data are not listed

Specimen pretreatment

The specimens were prepared into cell suspensions by the following procedure: disaggregated, rinsed in PBS, permeabilized with hypotonic 0.075 mol/L KCL at 37 °C for 30 min, fixed in fresh Carnoy's fixative (methanol/acetic acid, 3/1) for 30 min, and stored at 4 °C. Before hybridization, fixed cell suspensions were dropped onto cool wet slides and allowed to air dry overnight.

Fish

Chromosome enumeration probes (CEPs) 3, 6, 8, 11, 12, and 17 were purchased from ATCC (USA). CEPs 2, 7, and 9 were a gift from Professor Shu-Jun Cheng in the Department of Etiology and Carcinogenesis, Cancer Institute/Hospital, PUMC & CAMS. The probes were labeled using the BioPrime DNA labeling system (Invitrogen, Inc.) with cy3-dUTP, cy5-dUTP, and Spectrum-Green-dUTP. Multicolor FISH was performed on the specimens. We also performed independent verifications using single-color FISH in some cases. The slides were incubated with RNaseA (Sigma, 0.1 g/L in 2× SSC) at 37 °C for 45 min and with pepsin (Sigma, 0.1 g/L in 0.01 N HCl) at 37 °C for 15 min. The slides were fixed in paraformaldehyde (1 % in PBS/50 mM $MgCl_2$) for 10 min. Then the slides were denatured in 70 % formamide/2× SSC (PH 7.0) at 73 °C for 3 min and were immersed twice in 2× SSC at 4 °C for 5 min. Each of the above steps was followed by sequential dehydration in 75, 85, and 100 % ethanol solutions. The two or three probes with different fluorescent dyes mixed, co-precipitated with human Cot-1 DNA (Invitrogen, Inc.) and salmon sperm DNA (Sigma, Inc.), were dissolved in hybridization buffer composed of 60 % formamide, 10 % DS, 1 % Tween-20, and 2× SSC. After denatured at 73 °C for 8 min, the probes were applied to the target areas on each slide, covered with a coverslip, and sealed with rubber cement. The slides were hybridized overnight at 37 °C, and posthybridization washing was performed in 50 % formamide solution/2× SSC (pH 7.0) at 43 °C for 15 min. The slides were washed twice in 2× SSC for 3 min and then dehydrated in serial ethanol solutions. Nuclei were counterstained with 4′6-diamidino-2-phenylindole dihydrochloride (DAPI).

FISH scoring

Gray images of FISH were captured with a cooled charged-coupled device camera (Princeton, Inc.) equipped with an Opton fluorescence microscope. The images were analyzed using the MetaMorph Imaging System software (Universal Imaging Corporation). At least 100 interphase cell nuclei of each tumor sample were analyzed. To avoid bias, the results of FISH were scored blindly by two independent observers with no information of the clinical data. The FISH results of each case were analyzed only when the two observations were consistent. Nuclei with only one or more than two signals for a certain probe were assessed as aneuploidy. Overlapping, damaged, or smeared nuclei were excluded from analyses. Two signals that were situated within a distance of less than one signal size were counted as one signal.

The optimal cut-off value for each probe to distinguish normal cells from malignant cells was identified from its receiver operating characteristics (ROC) curves (Fig. 1). A specimen was classified as positive for cancer if it contained aneuploidy of one probe more than its cut-off value. Subsequently, combinations of three probes were analyzed, and positive cases were defined as aneuploidies of at least two probes.

Statistical analysis

Statistical analysis was performed using PASW Statistics 18 (SPSS Inc., Chicago). McNemar test was applied to compare sensitivity and specificity between cytology alone and combined with FISH detection. Associations between probe aneuploidy and clinical–pathological parameters were assessed using the Mann–Whitney test and Kruskal–Wallis test. For survival analysis, Kaplan–Meier survival curves were constructed, and differences between them were tested by the log-rank test. Follow-up data were updated in August 2011. Overall survival (OS) was defined as the time between the surgery date and the date of death from lung cancer or the date of last contact. The data of patients who were alive at the end of the study were censored. All P values were two-sided and were considered statistically significant if $P < 0.05$.

Results

Patient characteristics

Of the 74 tumor tissues collected in our study, 46 (62.2 %) were at early stage (stage I and stage II) with 19 SCCs and 27 ADCs. The bronchial brushings were collected from 59 SCCs, 34 ADCs, and 36 SCLCs. Sixty-six patients underwent surgery in Cancer Hospital, CAMS & PUMC, shortly after abnormal findings under bronchoscopy, and got their p-TNM. Twenty-four (36.3 %, 15 SCCs and 9 ADCs) patients exhibited early stage lung cancer. Detailed clinical and pathological characteristics are listed in Table 1. Forty-five bronchial brushings were from patients without pathological evidence of malignancy. The diagnoses were as follows: pneumonia ($n = 23$), chronic

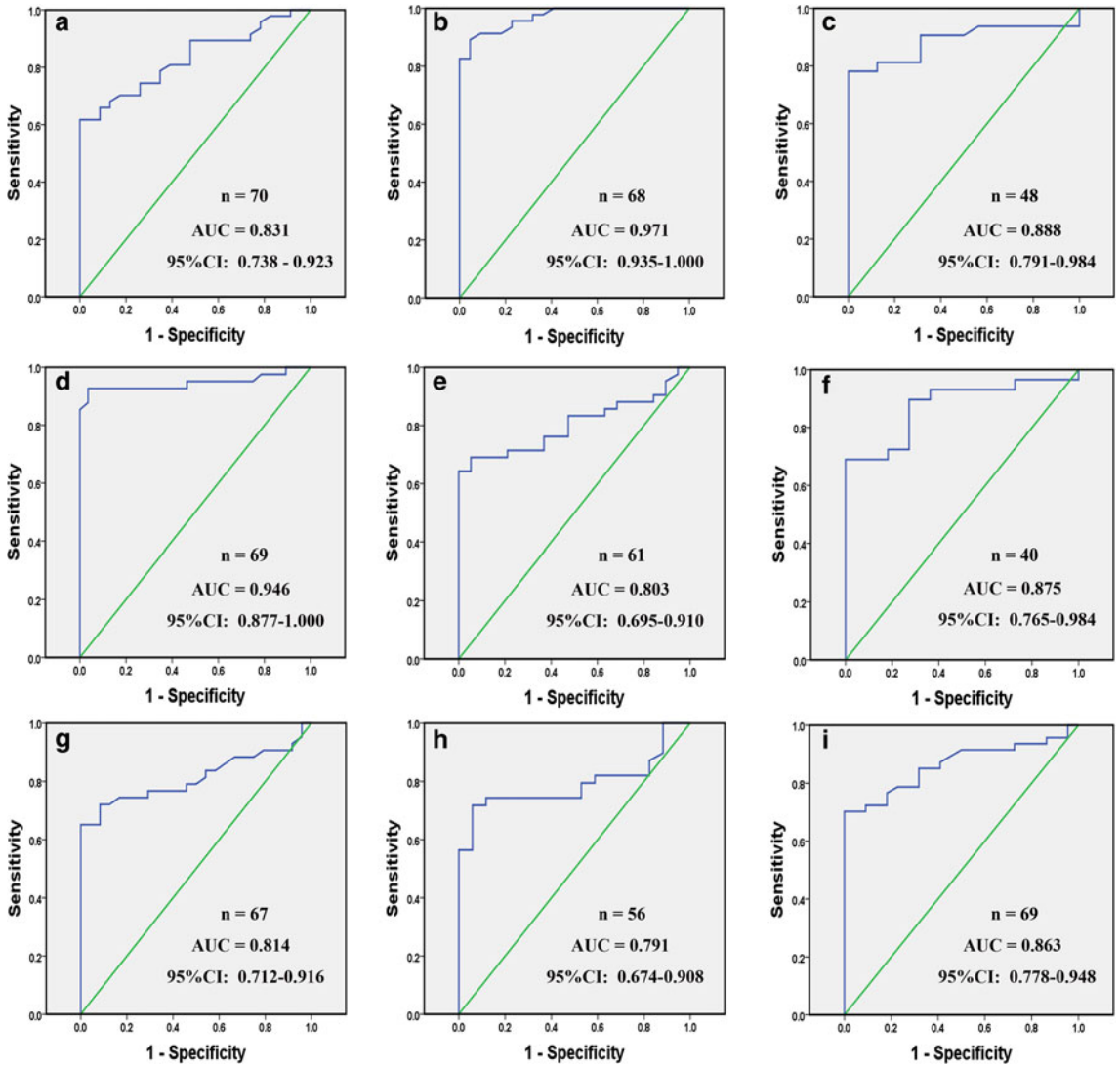

Fig. 1 ROC curves for CEP 2, 3, 6, 7, 8, 9, 11, 12, and 17 in 74 NSCLC tumor tissues and 32 surgical margin tissues. The determined cut-off values were 35.3, 14.6, 19.3, 11.7, 13.9, 20.2, 20.7, 17.1, and 21.2 % for CEP 2 (**a**), 3 (**b**), 6 (**c**), 7 (**d**), 8 (**e**), 9 (**f**), 11 (**g**), 12 (**h**), and 17 (**i**), respectively

granulomatous disease ($n = 11$), lung abscess ($n = 1$), infection ($n = 1$), sarcoidosis ($n = 1$), and indeterminate ($n = 8$).

Aneuploidy rates of the probes in tissue samples and bronchial brushings

Most of the aberrations for the nine probes found in both tumor tissues and bronchial brushings of pathologically confirmed lung cancer were chromosomal gains, including trisomy, tetrasomy, and polysomy. Monosomy and nullosomy were rarely detected (Fig. 2). Based on the ROC curves constructed by 74 NSCLC tumor tissues and 32 surgical margin tissues, the determined cut-off values were 35.3, 14.6, 19.3, 11.7, 13.9, 20.2, 20.7, 17.1, and 21.2 % for chromosome 2, 3, 6, 7, 8, 9, 11, 12, and 17, respectively. The aneuploidy rates were 61.7 % (29/47), 89.1 % (41/46), 80.0 % (24/30), 92.7 % (38/41), 65.0 % (26/40), 70.4 % (19/27), 66.7 % (28/42), 71.8 % (28/39), and 68.9 % (31/45) in tumor tissues and 29.3 % (24/82), 58.9 % (56/95), 33.3 % (19/57), 69.6 % (71/102), 67.0 % (63/94), 40.3 % (25/62), 38.0 % (19/50), 49.3 % (34/69), and 35.1 % (26/74) in bronchial brushings for chromosome 2, 3, 6, 7, 8, 9, 11, 12, and 17, respectively.

Sensitivity and specificity of a three-probe set for the detection of lung cancer

In the 129 bronchial brushings from patients with pathologically confirmed lung cancer, cytological examinations

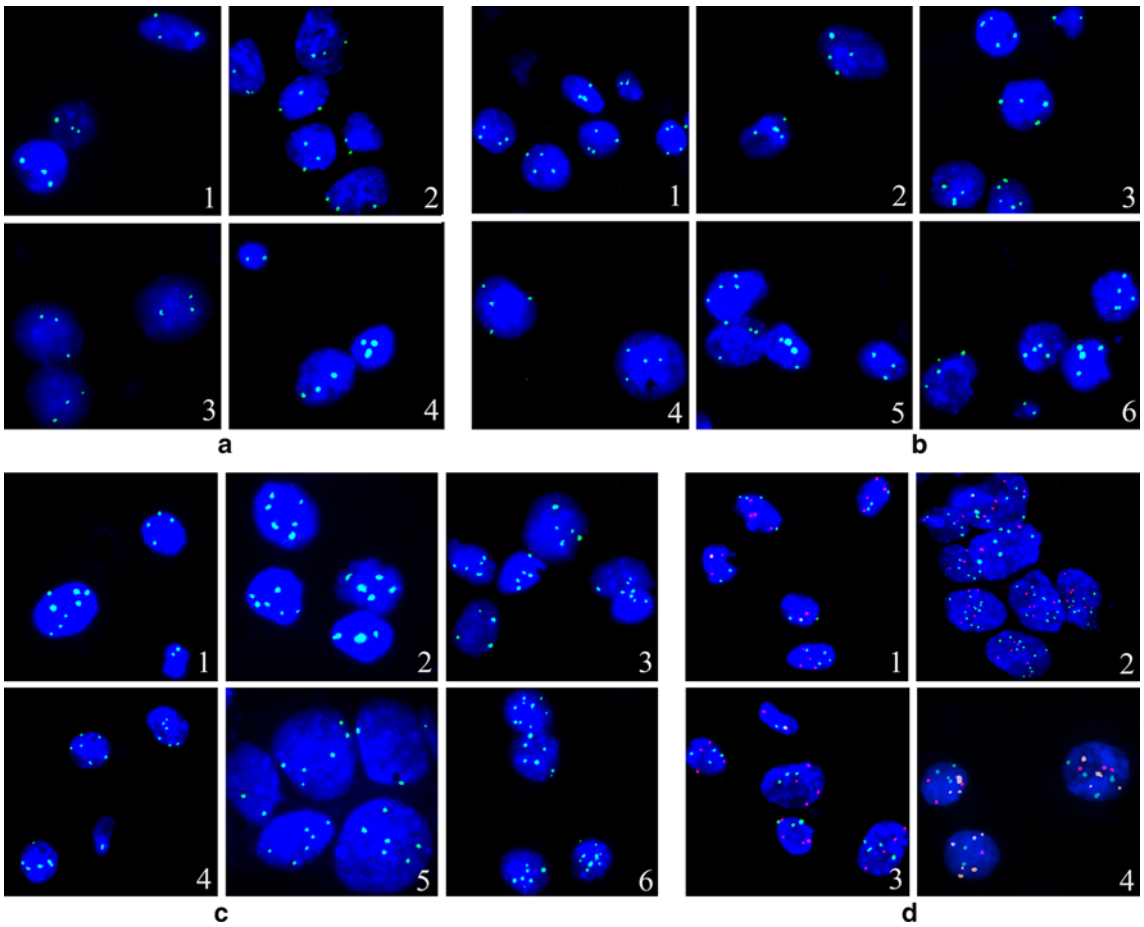

Fig. 2 Representative cases with trisomy, tetrasomy, and polysomy using different probes in bronchial brushings and tumor tissues. **a** Trisomy for CEP7 (*1*) and CEP8 (*2*) in bronchial brushing specimens and CEP9 (*3*) and CEP17 (*4*) in tumor tissues. **b** Tetrasomy for CEP7 (*1*), CEP8 (*2*), and CEP12 (*3*) in bronchial brushing specimens and CEP2 (*4*), CEP6 (*5*), and CEP11 (*6*) in tumor tissues. **c** Polysomy for CEP7 (*1*) and CEP12 (*2*) in bronchial brushings specimens and CEP2 (*3*), CEP6 (*4*), CEP9 (*5*), and CEP17 (*6*) in tumor tissues. **d** Aneuploidies detected by Multitarget FISH. *1* CEP7 (*Red*) and CEP3 (*Green*); *2*. CEP12 (*Red*) and CEP8 (*Green*); 3. CEP8 (*Red*) and CEP17 (*Green*); 4. CEP12 (*Red*), CEP11 (*Yellow*) and CEP7 (*Green*). FISH analyses (*1*) and (*2*) were performed in bronchial brushing specimens; *3* and *4* were performed in tumor tissues

revealed 98 positive and 31 negative (15 negative and 16 equivocal). No positive cytology results were observed in all the 45 bronchial brushings from patients with benign lung lesions. Therefore, the sensitivity of cytology diagnosis was 76.0 %, and the specificity was 100 % in this series of bronchial brushings.

Among the three-probe combinations, 3+7+8 showed the highest positive rate in both sample types, with abnormality in tumor tissues of 89.5 % SCCs and 88.2 % ADCs, and in bronchial brushings of 81.6 % SCCs and 63.0 % ADCs. When combining FISH of the three-probe set with cytology, the sensitivity of bronchial brushing examination was 98.2 % in SCCs, significantly higher than 78.0 % of cytology alone ($P = 0.008$). In addition, the combined sensitivity of FISH and cytology brushings for late-stage lesions was significantly better than cytology alone ($P = 0.016$). Significantly more central (95.6 vs. 82.1 %; $P = 0.008$) and peripheral tumors (93.3 vs. 58.7 %; $P = 0.008$) were identified using the combination of FISH and cytology compared to cytology alone. More importantly, in patients with pathologically positive and cytologically negative or equivocal lesions, the probe set demonstrated aneuploidy in 72.7 % of cases, that is, FISH detected an additional 16 of the 22 lung cancers that were undetectable using cytology alone (Table 2). When analyzing the sensitivity and specificity of this probe set in the 129 bronchial brushings with pathologically proven lung cancer and 45 bronchial brushings from patients with benign lung lesions, we found that the combination of cytology and FISH significantly increased the sensitivity for lung cancer detection (95.5 vs. 76.0 %; $P = 0.00003$). No significant decrease in specificity compared to cytology alone was observed (94.6 vs. 100.0 %; $P = 0.500$).

Table 2 Sensitivity of a three-probe set 3+7+8 in bronchial brushings by histological subtype of tumors

Probe set	Cytology	FISH	*P* value[a]	Combined	*P* value[b]
Total	76.0 % (98/129)	66.7 % (64/96)	0.165	95.0 % (114/120)	0.00003
SCC	78.0 % (46/59)	81.6 % (31/38)	0.791	98.2 % (54/55)	0.008
SCLC	77.8 % (28/36)	51.6 % (16/31)	0.035	91.2 % (31/34)	0.250
ADC	70.6 % (24/34)	63.0 % (17/27)	0.581	93.5 % (29/31)	0.063
LC^{NC}	–	72.7 % (16/22)	–	–	–
Early stage	66.7 % (16/24)	58.8 % (10/17)	0.508	95.0 % (19/20)	0.250
Late stage	73.8 % (31/42)	85.7 % (24/28)	0.344	97.4 % (38/39)	0.016
Central tumors	82.1 % (78/95)	67.6 % (48/71)	0.054	95.6 % (86/90)	0.008
Peripheral tumors	58.7 % (20/34)	64.0 % (16/25)	1.000	93.3 % (28/30)	0.008

Specificity between combined cytology/FISH and cytology was of no significant difference (see text)

LC^{NC} Lung cancer with negative cytology

[a] Difference in sensitivity between FISH and cytology

[b] Difference in sensitivity between combined cytology/FISH and cytology alone

Relationships between the aneuploidy rates of the probes and clinicopathological features and their impact on overall survival

We evaluated associations between the aneuploidy rates of the probes and clinicopathological parameters and analyzed their impact on the overall survival (OS) of patients. In bronchial brushings, higher aneuploidy rate of CEP8 was detected more in male patients ($P = 0.002$). The high aneuploidy rate of the 3+7+8 probe set was positively associated with tumor type (81.6, 63.0, and 51.6 % in SCC, ADC, and SCLC, respectively, $P = 0.029$) and NSCLC stage (58.8 and 85.7 % in early and late stages, respectively, $P = 0.044$; Table 3). Kaplan–Meier curves indicated that the ADC patients with high aneuploidy rate of CEP3 in tissue samples exhibited a poorer OS compared to those with a low aneuploidy rate of the probe ($P = 0.016$, Fig. 3).

Discussion

It has been shown that malignant neoplasms are characterized by widespread gains and losses of genetic material at the chromosome level. Lung cancer is characterized by complicated aberrations that affect most chromosomes (Berrieman et al. 2004; Varella-Garcia et al. 2007; Aviel-Ronen et al. 2008; Lockwood et al. 2008). The present study indicated that all of the nine tested probes revealed aneuploidy in tumor specimens with higher frequencies than the bronchial brushing specimens. The main reason might be the numbers of malignant tumor cells, because brushing specimens included more nonmalignant diploid cells (e.g., epithelial cells, stromal cells, and inflammatory cells) compared to operative tumor tissues. The optimal cut-off values for the definition of aneuploidy for each probe in our study were determined from the ROC curves, which are in accordance with previous reports (Sokolova et al. 2002; Nakamura et al. 2005; Li et al. 2006).

FISH has been widely applied in the diagnosis of cancers as an ancillary tool to cytology (Halling and Kipp 2007). Many groups have used different sets of probes for the detection of lung cancer (Halling et al. 2006; Nakamura et al. 2005; Barkan et al. 2005; Li et al. 2006; Fan et al. 2010). The commercially available LAVysion multi-target probe set for the detection of lung cancer contains four probes that are specific for chromosome 6, chromosomal regions 5p15, 7p12 (EGFR), and 8q24 (c-Myc). Some investigators demonstrated that the LAVysion probe set was more sensitive than conventional cytology (Bubendorf et al. 2005; Halling et al. 2006; Varella-Garcia et al. 2004). However, Barkan et al. found that the LAVysion probe set was positive in 57 % of tumor tissues, but negative in all bronchial brushings from lung cancer patients. A combination of 3p22.1 and 10q22 probes predicted 100 % of lung cancers in bronchial brushings ipsilateral to the tumor, but the sample size was small (Barkan et al. 2005). In the present study, we found that the three-probe set, 3+7+8, was a good candidate probe combination for the detection of Chinese lung cancer in bronchial brushing specimens. Our model has the advantage of using only three probes compared to the commercial kit (four probes). Additionally, our data were collected from observations in a relatively large sample of both tumor tissues and bronchial brushings.

Increased sensitivity generally accompanies a decrease in specificity. The optimal biomarker should exhibit the best balance between sensitivity and specificity. The combination of cytology with FISH using our 3+7+8 three-probe set significantly increased the sensitivity of

Table 3 Correlation between aneuploidy rate of the probe (set) and clinicopathological parameters in bronchial brushings

Parameter	CEP3	CEP7	CEP8	3+7+8
Sex				
Male	59.0 % (46/78)	69.2 % (54/78)	74.0 % (57/77)	67.5 % (52/77)
Female	58.8 % (10/17)	70.8 % (17/24)	35.3 % (6/17)	63.2 % (12/19)
P value[a]	0.991	0.882	0.002	0.719
Age				
≥60	62.9 % (22/35)	63.4 % (26/41)	63.9 % (23/36)	66.7 % (24/36)
<60	56.7 % (34/60)	73.8 % (45/61)	69.0 % (40/58)	66.7 % (40/60)
P value[a]	0.556	0.267	0.613	1.000
Tumor type				
SCC	66.7 % (28/42)	74.4 % (32/43)	71.4 % (30/42)	81.6 % (31/38)
ADC	56.0 % (14/25)	76.7 % (23/30)	60.9 % (14/23)	63.0 % (17/27)
SCLC	50.0 % (14/28)	55.2 % (16/29)	65.5 % (19/29)	51.6 % (16/31)
P value[b]	0.363	0.136	0.676	0.029
Gross pathology				
Central-type	62.0 % (44/71)	67.1 % (49/73)	70.0 % (49/70)	67.6 % (48/71)
Peripheral type	50.0 % (12/24)	75.9 % (22/29)	58.3 % (14/24)	64.0 % (16/25)
P value[a]	0.305	0.389	0.297	0.744
Grade				
G1 + G2	70.6 % (12/17)	57.9 % (11/19)	64.7 % (11/17)	68.8 % (11/16)
G3	52.0 % (13/25)	80.0 % (24/30)	62.5 % (15/24)	70.8 % (17/24)
P value[a]	0.234	0.099	0.887	0.889
NSCLC stage				
I + II	52.6 % (10/19)	63.2 % (12/19)	70.6 % (12/17)	58.8 % (10/17)
III + IV	72.4 % (21/29)	84.8 % (28/33)	64.3 % (18/28)	85.7 % (24/28)
P value[a]	0.166	0.077	0.667	0.044
T status				
T1 + T2	62.1 % (18/29)	74.2 % (23/31)	72.4 % (21/29)	71.4 % (20/28)
T3 + T4	66.7 % (12/18)	78.9 % (15/19)	57.1 % (8/14)	81.3 % (13/16)
P value[a]	0.752	0.705	0.322	0.474
N status				
N0	69.2 % (9/13)	69.2 % (9/13)	63.6 % (7/11)	75.0 % (9/12)
N1–3	61.8 (21/34)	78.4 % (29/37)	68.8 % (22/32)	75.0 % (24/32)
P value[a]	0.637	0.511	0.758	1.000
M status				
M0	64.3 % (27/42)	79.1 % (34/43)	69.2 % (27/39)	78.9 % (30/38)
M1	66.7 % (4/6)	66.7 % (6/9)	50.0 % (3/6)	57.1 % (4/7)
P value[a]	0.910	0.426	0.358	0.223

[a] Mann–Whitney test

[b] Kruskal–Wallis test

bronchial brushing examination for the detection of lung cancer ($P = 0.00003$). Compared to cytology alone, this increased sensitivity was achieved, and high specificity was maintained (94.6 %). Similarly high positive rates that were detected in tumor tissues further supported that these probes may serve as potential hallmarks for lung cancer. Nevertheless, the lower number of controls in our research may have led to the higher specificity. FISH provides a simple, fast, and reliable means to assess genetic instability in cancer, and it can improve the efficacy of cytology detection (Fox et al. 1995). The increased capacity of FISH to detect disease earlier than cytology could improve overall survival of patients. Our study also demonstrated that in detecting peripheral tumors, the combination of FISH and cytology significantly improved the diagnostic sensitivity as compared to cytology alone.

When considering clinical parameters, FISH with our three-probe set improved the cytology diagnosis of late-stage cancer patients. This probe set was more suitable for SCCs, but not for ADCs and SCLCs, which was consistent with the positive associations between the high aneuploidy rate of the probe set and NSCLC stage (58.8 and 85.7 % in early and late stages, respectively, $P = 0.044$) and tumor type (81.6, 63.0, and 51.6 % in SCC, ADC, and SCLC,

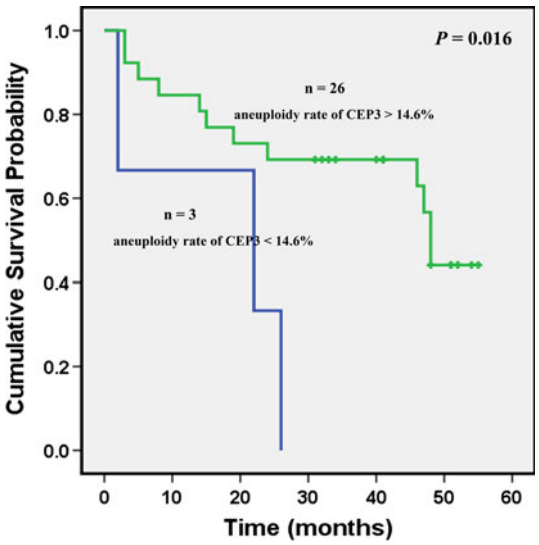

Fig. 3 Relationship between the aneuploidy rate of CEP3 in tissue samples and overall survival of ADC patients. Kaplan–Meier curves showing that ADC patients with high aneuploidy rate of CEP3 had a poorer OS compared to those with low aneuploidy rate of the probe ($P = 0.016$)

respectively, $P = 0.029$). Future studies are required to detect other molecular probes in the latter two tumor types. Our results are consistent with previously published reports. Several comparative genomic hybridization studies have illustrated that lung cancers carry distinct patterns of chromosomal imbalances that are associated with tumor progression (Ried et al. 1994; Goeze et al. 2002; Petersen et al. 2000). Petersen et al. (Petersen et al. 1997b) suggested that higher DNA content and chromosomal gains in SCC were more frequent than other subtypes of lung cancer, and SCLC was characterized by a high incidence of chromosomal losses. Distinct differences between SCC and ADC have also been illustrated from molecular analyses (Petersen et al. 1997a; Petersen 2011).

Cytology is difficult to distinguish atypical cells from malignant cells. Savic et al. reported a higher sensitivity (79 %) in equivocal cytology cases using LAVysion set (Savic et al. 2006). They first determined the exact location of the atypical cells on the specimen slides by Papanicolaou staining. The slides were hybridized with the LAVysion set, and the equivocal cells were relocated and scored. This approach may significantly improve diagnosis by decreasing the influence of background cells. Our data with a three-probe set in cytology equivocal cases support the usefulness of FISH as a complement for cancer detection. Future studies should validate the probe set with relocation FISH onto equivocal specimens.

Our study also showed that in tissue samples, ADC patients with high aneuploidy rate of CEP3 had a poorer OS. Larger studies with extended follow-ups are required to determine whether a higher aneuploidy rate of CEP3 in bronchial brushings indicates a poorer prognosis.

In summary, our data indicate that FISH with optimal probes performed on cytology preparations is useful for the diagnosis of lung cancer, especially in conjunction with cytology. The 3+7+8 three-probe set has the potential value for the detection of squamous cell lung cancer with bronchial brushings, to which additional independent and prospective validation studies are needed.

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Conflict of interest The authors declare no conflict of interest.

References

Agarwal M, Brahmanday G, Chmielewski GW, Welsh RJ, Ravikrishnan KP (2010) Age, tumor size, type of surgery, and gender predict survival in early stage (stage I and II) non-small cell lung cancer after surgical resection. Lung Cancer 68(3):398–402

Aviel-Ronen S, Coe BP, Lau SK, da Cunha Santos G, Zhu CQ, Strumpf D, Jurisica I, Lam WL, Tsao MS (2008) Genomic markers for malignant progression in pulmonary adenocarcinoma with bronchioloalveolar features. Proc Natl Acad Sci U S A 105(29):10155–10160

Balsara BR, Testa JR (2002) Chromosomal imbalances in human lung cancer. Oncogene 21(45):6877–6883

Barkan GA, Caraway NP, Jiang F, Zaidi TM, Fernandez R, Vaporcyin A, Morice R, Zhou X, Bekele BN, Katz RL (2005) Comparison of molecular abnormalities in bronchial brushings and tumor touch preparations. Cancer 105(1):35–43

Berrieman HK, Ashman JN, Cowen ME, Greenman J, Lind MJ, Cawkwell L (2004) Chromosomal analysis of non-small-cell lung cancer by multicolour fluorescent in situ hybridisation. Br J Cancer 90(4):900–905

Bubendorf L, Muller P, Joos L, Grilli B, Vogel S, Herzog M, Barascud A, Feichter G, Dalquen P, Tamm M (2005) Multitarget FISH analysis in the diagnosis of lung cancer. Am J Clin Pathol 123(4):516–523

Choma D, Daures JP, Quantin X, Pujol JL (2001) Aneuploidy and prognosis of non-small-cell lung cancer: a meta-analysis of published data. Br J Cancer 85(1):14–22

Fan YB, Ye L, Wang TY, Wu GP (2010) Correlation between morphology and human telomerase gene amplification in bronchial brushing cells for the diagnosis of lung cancer. Diagn Cytopathol 38(6):402–406

Fox JL, Hsu PH, Legator MS, Morrison LE, Seelig SA (1995) Fluorescence in situ hybridization: powerful molecular tool for cancer prognosis. Clin Chem 41(11):1554–1559

Goeze A, Schluns K, Wolf G, Thasler Z, Petersen S, Petersen I (2002) Chromosomal imbalances of primary and metastatic lung adenocarcinomas. J Pathol 196(1):8–16

Halling KC, Kipp BR (2007) Fluorescence in situ hybridization in diagnostic cytology. Hum Pathol 38(8):1137–1144

Halling KC, Rickman OB, Kipp BR, Harwood AR, Doerr CH, Jett JR (2006) A comparison of cytology and fluorescence in situ hybridization for the detection of lung cancer in bronchoscopic specimens. Chest 130(3):694–701

Li R, Liu Z, Fan T, Jiang F (2006) A novel multiple FISH array for the detection of genetic aberrations in cancer. Lab Invest 86(6):619–627

Lockwood WW, Chari R, Coe BP, Girard L, Macaulay C, Lam S, Gazdar AF, Minna JD, Lam WL (2008) DNA amplification is a ubiquitous mechanism of oncogene activation in lung and other cancers. Oncogene 27(33):4615–4624

Mazzone P, Jain P, Arroliga AC, Matthay RA (2002) Bronchoscopy and needle biopsy techniques for diagnosis and staging of lung cancer. Clin Chest Med 23(1):137–158

Nakamura H, Aute I, Kawasaki N, Taguchi M, Ohira T, Kato H (2005) Quantitative detection of lung cancer cells by fluorescence in situ hybridization: comparison with conventional cytology. Chest 128(2):906–911

Panani AD, Roussos C (2006) Cytogenetic and molecular aspects of lung cancer. Cancer Lett 239(1):1–9

Petersen I (2011) The morphological and molecular diagnosis of lung cancer. Dtsch Arztebl Int 108(31–32):525–531

Petersen I, Bujard M, Petersen S, Wolf G, Goeze A, Schwendel A, Langreck H, Gellert K, Reichel M, Just K, du Manoir S, Cremer T, Dietel M, Ried T (1997a) Patterns of chromosomal imbalances in adenocarcinoma and squamous cell carcinoma of the lung. Cancer Res 57(12):2331–2335

Petersen I, Langreck H, Wolf G, Schwendel A, Psille R, Vogt P, Reichel MB, Ried T, Dietel M (1997b) Small-cell lung cancer is characterized by a high incidence of deletions on chromosomes 3p, 4q, 5q, 10q, 13q and 17p. Br J Cancer 75(1):79–86

Petersen S, Aninat-Meyer M, Schluns K, Gellert K, Dietel M, Petersen I (2000) Chromosomal alterations in the clonal evolution to the metastatic stage of squamous cell carcinomas of the lung. Br J Cancer 82(1):65–73

Ried T, Petersen I, Holtgreve-Grez H, Speicher MR, Schrock E, du Manoir S, Cremer T (1994) Mapping of multiple DNA gains and losses in primary small cell lung carcinomas by comparative genomic hybridization. Cancer Res 54(7):1801–1806

Savic S, Glatz K, Schoenegg R, Spieler P, Feichter G, Tamm M, Bubendorf L (2006) Multitarget fluorescence in situ hybridization elucidates equivocal lung cytology. Chest 129(6):1629–1635

Siegel R, Ward E, Brawley O, Jemal A (2011) Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. CA Cancer J Clin 61(4):212–236

Sokolova IA, Bubendorf L, O'Hare A, Legator MS, Jacobson KK, Grilli BSB, Dalquen P, Halling KC, Tamm M, Seelig SA, Morrison LE (2002) A fluorescence in situ hybridization-based assay for improved detection of lung cancer cells in bronchial washing specimens. Cancer 96(5):306–315

Tonon G, Wong KK, Maulik G, Brennan C, Feng B, Zhang Y, Khatry DB, Protopopov A, You MJ, Aguirre AJ, Martin ES, Yang Z, Ji H, Chin L, Depinho RA (2005) High-resolution genomic profiles of human lung cancer. Proc Natl Acad Sci U S A 102(27):9625–9630

Varella-Garcia M, Kittelson J, Schulte AP, Vu KO, Wolf HJ, Zeng C, Hirsch FR, Byers T, Kennedy T, Miller YE, Keith RL, Franklin WA (2004) Multi-target interphase fluorescence in situ hybridization assay increases sensitivity of sputum cytology as a predictor of lung cancer. Cancer Detect Prev 28(4):244–251

Varella-Garcia M, Chen L, Powell RL, Hirsch FR, Kennedy TC, Keith R, Miller YE, Mitchell JD, Franklin WA (2007) Spectral karyotyping detects chromosome damage in bronchial cells of smokers and patients with cancer. Am J Respir Crit Care Med 176(5):505–512

Wen C, Dehnel T (2011) China wrestles with lung cancer. Lancet Oncol 12(1):15

Yendamuri S, Vaporciyan AA, Zaidi T, Feng L, Fernandez R, Bekele NB, Hofstetter WL, Jiang F, Mehran RJ, Rice DC, Spitz MR, Swisher SG, Walsh GL, Roth JA, Katz RL (2008) 3p22.1 and 10q22.3 deletions detected by fluorescence in situ hybridization (FISH): a potential new tool for early detection of non-small cell lung Cancer (NSCLC). J Thorac Oncol 3(9):979–984