Chromosomal Abnormalities in Non-Small Cell Lung Carcinomas and in Bronchial Epithelia of High-Risk Smokers Detected by Multi-Target Interphase Fluorescence *in Situ* Hybridization

Maura Santos Romeo,* Irina A. Sokolova,[†]
Larry E. Morrison,[†] Chan Zeng,[‡] Anna E. Barón,[‡]
Fred R. Hirsch,* York E. Miller,^{*§}
Wilbur A. Franklin,[¶] and Marileila Varella-Garcia*

From the Departments of Medicine,* Preventive Medicine and Biometrics,[‡] and Pathology,[¶] School of Medicine, University of Colorado Health Sciences Center, Denver, Colorado; the Department of Medicine,[§] Denver Veterans Administration Medical Center, Denver, Colorado; and Vysis, Inc.,[†] Downers Grove, Illinois

Human lung carcinogenesis is accompanied by complex chromosomal changes that may be detected in interphase cells by fluorescence *in situ* hybridization (FISH) assay using recently developed multitarget DNA probes. Touch preparations of 20 non-small cell lung carcinomas, sputum specimens from 3 patients with lung cancer and from 11 ex-smokers without lung cancer, and cultured benign bronchial epithelium of 42 high-risk smokers, 9 of whom had concurrent invasive carcinoma, were tested using a four-color FISH probe (LAVysion) targeting centromere 6, 5p15.2, 7p12 (EGFR), and 8q24 (MYC). Significantly high frequencies of abnormal cells were found in each of the 20 NSCLC (100%) and in the 3 sputum specimens from lung cancer patients. None of the cytologically normal sputa contained FISH abnormalities. Cultured bronchial epithelial cells from 11 of 42 patients (26%) were abnormal for at least one probe. Abnormal FISH patterns had no association with gender, presence of tumor or histology. Multicolor FISH can readily detect chromosomal abnormalities in imprints and sputa from lung carcinomas. Chromosomal aneusomy is also frequent in bronchial epithelial cells from long-term smokers. The prognostic significance of the multicolor LAVysion FISH probe set should be validated in a controlled clinical trial. (*J Mol Diagn* 2003, 5:103–112)

Documented genetic abnormalities associated with initiation and progression of lung cancer include point mutations, allelic loss and methylation of tumor suppressor genes^{1,2}; however, to date, none of these abnormalities have proven successful as biomarkers for early detection

or in predicting who will get lung cancer and who might benefit from early intervention. Obstacles to the clinical application of these molecular lesions as biomarkers are many and have included the heterogeneity of lung tumors, infrequency of individual markers in the lung cancer population, remoteness from imminent malignant transformation and, finally, the lack of expression in accessible specimens such as sputum.

As in many other solid tumors, chromosomal rearrangements and aneusomy involving multiple chromosomes are frequently detected by interphase FISH assays in lung cancer.^{3–5} The inability to maintain consistent chromosomal structure and number through successive cell divisions is known as chromosomal instability. Similar patterns of chromosomal aneusomy have been found in both invasive tumors and in pre-malignant lesions of the same patients, although in fewer cells and involving fewer chromosomes in nonmalignant specimens.⁶ These findings support the hypothesis that chromosomal instability occurs before the invasive stage of the carcinogenic process.

The effectiveness of cytogenetic methods as adjunct diagnostic and prognostic tools in solid tumors has been difficult to demonstrate, mainly due to difficulty in obtaining fresh tissue, low proliferative rate of these lesions *in vitro*, and lack of specificity of the multiple changes usually detected. Interphase analysis using fluorescence *in situ* hybridization (FISH)-based techniques overcome many of these problems and the applicability of interphase FISH in cancer has been confirmed in the past few years. For instance, multitargeted FISH probes have been demonstrated as highly sensitive and specific for diagnosis of breast cancer in fine needle aspirate⁷ and superior to conventional cytology for the detection of urothelial carcinoma in urine specimens.⁸

Supported in part by the National Cancer Institute grants CCSG P30-CA46934, Specialized Program of Research Excellence in Lung Cancer P01-CA58187, and Early Detection Research Network U01-CA85070. M. S. Romeo was a post-doctoral fellow of the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil).

Accepted for publication February 11, 2003.

Address reprint requests to Wilbur A. Franklin, M.D., University of Colorado Health Sciences Center, Campus Box B216, 4200 East 9th Avenue, Denver CO 80262. E-mail: wilbur.franklin@uchsc.edu.

Despite the high frequency of aneusomy in lung cancer FISH has not yet been fully exploited for early detection and monitoring of this tumor type, in part because of the unavailability of validated probes for application specifically to lung carcinoma. In the present study, four DNA targets representing genes or chromosomal regions frequently reported as abnormal in non-small cell lung carcinomas (NSCLC) were tested in a multicolor FISH panel. The sensitivity and specificity of this multicolor FISH probe in detecting tumor cells was evaluated and its effectiveness in detecting abnormalities in tumor, nonmalignant bronchial epithelium (BE), and sputum was assessed.

Materials and Methods

Specimens

Imprints (touch preparations) were prepared from 10 squamous cell (SCC) and 10 non-squamous cell carcinomas including 7 bronchioloalveolar carcinomas (BAC) and 3 adenocarcinomas (ADC) at the time of surgical resection with informed consent according to a research protocols approved by the Colorado Multiple Institutional Review Board (COMIRB). As a control, touch preparations from 5 normal lung, 3 bronchus, and 2 tonsil specimens were used. Imprints were obtained according to protocol described in Varella-Garcia et al.⁹ The freshly resected tumor and normal tissues were imprinted on silanized slides, fixed in methanol and stored at -80°C until use.

The sputum specimens were obtained from high-risk smokers enrolled in fluorescence bronchoscopy trials (see below) or preoperatively from patients with lung cancer according to COMIRB-approved research protocols. All participants had a smoking history of more than 30 pack-years. Sputum samples were obtained from 3 patients diagnosed with SCC and from 11 cancer-free ex-smokers. Sputa were initially fixed in Saccomanno's solution at the collection time and stored at room temperature for up to 18 months. Approximately 1 ml of sputum suspension was diluted with 5 ml of 1X PBS, centrifuged, and the cell pellet was resuspended in 15 minutes at room temperature in 2.5 ml of HBSS containing 10 mmol/L ethylenediamine tetracetic acid and 50 μl of dithiothreitol. After another centrifugation, the cell pellet was fixed in Carnoy's fixative and the suspension was dropped onto glass slides with adjustments for proper cellularity when necessary.

To evaluate the feasibility of detecting chromosomal abnormalities by multicolor FISH in biopsies of BE from high-risk smokers without having to address fixation and tissue sectioning problems associated with *in situ* evaluation, we chose to prepare monolayers of cultured BE. All of the 42 individuals contributing benign BE samples to this study were or had been heavy smokers. Eighteen were former smokers and 24 were current smokers. Pack-year smoking histories ranged from 30 to more than 150 pack-years with a mean of 68.5. Subjects ranged in

age from 45 to 77 years; 28 were male and 14 were female.

BE was obtained in two ways. The first set of BE samples was obtained from remnant bronchial tissue of 9 patients undergoing surgical resection for lung carcinoma at the University of Colorado Health Sciences Center and the Denver Veterans Administration Medical Center. Epithelia from bronchi distant from and uninvolved by tumor were digested with dispase (Becton Dickinson, Franklin Lakes, NJ) and allowed to attach to Biocoat T25 flasks in BEGM culture medium (Clonetics Inc., Walkersville, MD). They were then cultured to 90% confluence (about 4 days), passaged into a second Biocoat T25 flask, and split onto glass hybridization coverslips for a total of no more than 14 days in culture. Coverslips of cultured bronchial epithelial cell samples were fixed in Carnoy's solution with three changes of fixative solution and air-dried. Copious nonmalignant whole bronchial cells were obtained by this procedure. These cells formed a monolayer in which signal counts for individual cells could be directly analyzed without having to compensate for sectioned or overlapping nuclei that are found in tissue sections of bronchial mucosa.

The second set of specimens was obtained from 33 subjects enrolled in a fluorescence bronchoscopy clinical trial at the University of Colorado Health Sciences Center designed to evaluate the detectability of abnormal BE in high-risk smokers without carcinoma and to correlate the histological features of BE with biomarker expression. The trial and biopsy procedures were approved by COMIRB and biopsies were obtained for testing only with informed consent of the patient. Requirements for entry into this trial included a smoking history of more than 30 pack-years, evidence of airway obstruction with FEV_1 less than 70% of the predicted value and moderate dysplasia or worse on sputum cytology.

Subjects meeting entry criteria were offered fluorescence bronchoscopy using a Xillix laser-induced fluorescence emission (LIFE) bronchoscope and were biopsied at several suspicious and normal appearing sites (range, 3–42).¹⁰ One or two of the biopsies were explanted onto a T25 culture flask containing BEGM medium (Clonetics, Inc., Walkersville, MD) and epithelial cells allowed to grow from the explant to a diameter of 1 cm (10 days). Cells were then passaged into a second T25 flask and grown to approximately 90% confluence. The culture cells were again split onto glass hybridization coverslips to perform FISH studies. The total time from biopsy date to cell harvest was no more than 14 days. BE used as controls were obtained from Clonetics (Normal Human Epithelial Cells CC2540, Lots # 0F0387 and # 0F1131) and from a 48-year-old, never-smoker, healthy female donor. Primary cultures processed in this way grow as substrate-adherent monolayers which are 100% cytokeratin positive on immunohistochemical staining.¹¹ Coverslips of cultured BE samples were fixed in Carnoy's solution with three changes of fixative solution, air-dried and stored at -80°C until use.

All but 2 to 3 biopsies from each patient were fixed in formalin and processed by conventional methods for hematoxylin and eosin staining. Histology of each biopsy

Figure 1. FISH patterns identified with the LAVysion probe. Sequences from chromosome 5p15.2 are labeled in green, from 6p11-q11 in aqua, from 7p12 in red and from 8q24 in yellow. **A:** Nuclei from normal lung showing diploid pattern 2222 (2 copies of each target). **B:** Nucleus with pattern 7687 in BAC4. **C:** 7p12 (EGFR) amplification in BAC1. **D:** Bronchial nuclei with abnormal tetrasomic pattern 4444 from P14-RML. **E:** Aneusomic pattern 4433 in a bronchial cell from P35. **F:** Aneusomic pattern 2244 indicating gain of 7p12 and 8q24 sequences in a nuclei found in sputum from a SCC patient.

specimen was graded according to a modification of the World Health Organization Classification,¹² on the following eight-tiered scale: 1. Normal; 2. Basal cell hyperplasia; 3. Squamous metaplasia; 4. Mild dysplasia; 5. Moderate dysplasia; 6. Severe dysplasia; 7. Carcinoma *in situ*; 8. Invasive carcinoma. Three indices were calculated for each patient including average histology score of all biopsied sites, dysplasia index (number of sites with squamous metaplasia, dysplasia, or CIS/total number of biopsied sites $\times$ 100), and highest score (highest degree of dysplasia or malignancy). For statistical correlations (see below), specimens were grouped as normal (normal morphology and hyperplasia) or abnormal (metaplasia, dysplasia, CIS) for the histology at the site, the average histology score in all biopsied sites, and the worst score (highest degree of dysplasia or malignancy). According to the abnormality index (% of sites with metaplasia, dysplasia, or CIS/total number of sites), patients were grouped as $\leq 15\%$ and $>15\%$, following Soria et al.¹³

Specimen Processing and Fluorescence in Situ Hybridization Assays

Slides and coverslips were brought to room temperature in a dry atmosphere to minimize moisture condensation just before the FISH assays. They were then incubated in 2X SSC at 37°C for 30 minutes, dehydrated in ethanol series, and incubated in 70% glacial acetic acid for 1 minute. The specimens were then digested in 0.05 μ g/ml pepsin at 37°C for 5 minutes, and fixed in 1% neutral buffered formaldehyde at room temperature for 10 minutes. The FISH probe set, LAVysion (Vysis, Downers Grove, IL), consisted of one centromeric probe (6p11.1-q11, CEP6) labeled in SpectrumAqua, and three locus-specific probes for 5p15.2 (D5S23, D5S721), 7p12 (EGFR) and 8q24.12-q24.13 (C-MYC), respectively labeled in SpectrumGreen, SpectrumRed, and SpectrumGold. To select the FISH probes incorporated into

Table 1. Frequencies of Signals per Cell for Each DNA Probe Included in the LAVysion Assay in Touch Preparations of NSCLC and Normal Lung, Bronchial, and Tonsil Specimens

Histology	No.	5p15		CEP6		7p12		8q24	
		Average	SD	Average	SD	Average	SD	Average	SD
Normal	10	1.9	0.00	2.0	0.17	2.1	0.24	2.0	0.17
Non-SCC (BAC and ADC)	10	4.2	2.38	3.4	1.74	3.9	2.54	4.1	2.43
SCC	10	4.4	2.96	3.1	1.26	3.1	1.71	4.0	1.99

Frequencies are average and SD.

Table 2. Number of FISH Signals per Cell for Each DNA Target Tested and Frequencies of Abnormal Cells per Target of Cells with a Hyper-Aneusomic Pattern and a Clustered Amplification Pattern in the 20 NSCLC Specimens

NSCLC	Stage	5p15						CEP 6				
		% Ab	% HA	Range	Modal	Average	SD	% Ab	Range	Modal	Average	SD
BAC1	IA	70		1-4	4	3.3	1.07	71	1-5	4	3.4	1.17
BAC2	IIA	95		2-5	4	3.9	0.52	95	2-5	4	3.9	0.69
BAC3	IV	100		3-13	5	5.8	2.45	99	2-12	2	4.8	2.33
BAC4	II	96	15	2-13	5	7.3	2.40	92	2-12	5	5.5	1.91
BAC5	IB	5		1-3	2	2.0	0.24	5	1-3	2	2.0	0.27
BAC6	IB	97		2-10	5	5.5	1.37	48	1-5	2	2.5	0.80
BAC7	IB	24		2-4	2	2.2	0.57	11	1-4	2	2.1	0.48
ADC1	IA	10		1-4	2	2.0	0.44	8	1-4	2	2.1	0.47
ADC2	IIIA	60		2-10	2	3.6	1.80	84	2-5	2	3.0	1.35
ADC3	IIB	100		3-10	8	7.3	1.54	100	6-8	6	5.4	1.21
SCC1	IIB	61		1-8	2	3.3	2.02	61	1-5	2	2.7	1.03
SCC2	I	97		2-7	4	3.8	0.83	97	2-5	3	3.1	0.47
SCC3	IIA	44		1-6	2	2.8	1.01	30	1-5	2	2.3	0.59
SCC4	II	61		2-7	3	3.0	1.05	63	1-7	3	3.0	1.15
SCC5	IV	100	60	6-15	12	10.5	2.91	100	3-9	5	4.6	0.98
SCC6	II	64		2-7	2	3.4	1.39	62	2-5	2	3.0	1.01
SCC7	IB	48		2-6	2	2.6	0.87	26	1-4	2	2.3	0.64
SCC8	IIB	96	26	2-16	6	7.4	3.06	98	2-9	3	4.6	1.58
SCC9	IB	100		3-8	5	4.8	1.15	91	2-8	3	3.6	1.15
SCC10	IB	19		2-6	2	2.4	1.07	19	2-6	2	2.3	0.76

*Split signals may indicate double minutes.

Numbers of FISH signals per cell are range, mode, average and SD.

% Ab, Frequency of abnormal cells per target.

% HA, Frequency of hyper-aneusomic pattern.

% Amp, Frequency of clustered amplification pattern.

the LAVysion probe set, 26 different loci were tested against a collection of lung carcinomas. Final selection of the four probes was based on probe discrimination and complementation analyses. The final set showed excellent sensitivity when previously tested on a series of bronchial wash specimens.¹⁴ The probe set was applied to the slide or the coverslip, the hybridization area was sealed and co-denaturation was performed at 80°C for 8 minutes followed by incubation at 37°C for 24 hours in a humidified chamber. Posthybridization washes were performed consecutively in 50% formamide/2X SSC, 2X SSC and 2X SSC/0.1% NP-40, each of them at 46°C for 6 minutes. DAPI in Vectashield antifade (0.15 µg/ml) was applied as chromatin counterstain.

Microscopic Analysis

Analysis was performed on an Olympus BX-60 epifluorescence microscope equipped with the Quips XL genetic workstation (Applied Imaging, Santa Clara, CA). Fluorescence signals were scored using single-band pass filters for DAPI, SpectrumAqua, FITC, SpectrumGold and Texas Red. Dual-band (FITC/Texas red) and triple (DAPI/FITC/Texas Red) band pass filters were also used when convenient.

In the tumor specimens, a selection of approximately 100 irregularly shaped and stained nuclei, at least two times larger in area than the average nucleus of an epithelial cell, was scored. Amplification was defined by multiple signals clustered at a single chromosomal site in ≥10% of cells. Hyperaneusomy was defined as ≥9 copies of a single target in a dispersed pattern in ≥10% of cells. In each of the BE specimens, 200 consecutive

nuclei were scored. Sputum specimens submitted for the FISH assay were initially scanned under the DAPI filter for presence of epithelial cells with irregularly shaped and stained nuclei, and the fluorescent signals for each target were scored in these nuclei. All irregularly shaped and stained nuclei from non-blood cells present in the hybridization were analyzed. Representative images were acquired with a SenSys cooled CCD camera (Photometrics, Tucson, AZ) in monochromatic layers which subsequently were merged and processed by the SmartCapture software (Vysis, Inc.).

Statistical Analysis

χ² tests were used to compare the FISH patterns across levels of variables such as gender, smoking status, presence of tumor, and histology classification. One-way analysis of variance was used to compare variability among probes in the control specimens. To determine cut points for cultured BE, receiver operating characteristic (ROC) analysis was used.¹⁵ This method shows the trade-off between sensitivity and the false positive rate over a range of cutoff values for the percentage of abnormal cells in a specimen. Sensitivity is estimated as the percentage of tumor or high-risk sputum specimens with an abnormal cell percentage above a given cutoff. The false positive rate (100% specificity) is estimated as the percentage of normal tissue or cultured specimens with an abnormal cell percentage above the cutoff. To be conservative in estimating the percentage of abnormal specimens, we chose the optimal cutoff for the percentage of abnormal cells as the one that results in a 0% false positive rate, or, equivalently, 100% specificity.

Table 2. *Continued*

7p12							8q24						
% Ab	% HA	% Amp	Range	Modal	Average	SD	% Ab	% HA	% Amp	Range	Modal	Average	SD
77		32	2-8	4	4.1	1.38	76			2-8	3	3.5	1.12
97			2-6	3	3.6	0.74	95			2-6	4	4.0	0.79
100	40		3-15	4	7.6	3.61	99	40		2-21	5	8.8	4.43
99	15		2-10	4	7.3	2.14	96			2-10	5	5.2	1.63
10			2-4	2	2.1	0.37	7			2-4	2	2.1	0.35
96			2-10	4	4.5	1.31	96			2-7	4	4.2	1.01
23			2-5	2	2.5	0.81	20			1-5	2	2.3	0.72
94			2-8	4	4.0	1.31	21			2-6	2	2.4	0.85
100			3-6	3	4.6	1.92	90			2-9	3	3.7	1.47
100			3-10	8	7.9	1.33	100			3-8	6	5.8	0.80
75			2-7	3	3.2	1.09	57			2-10	2	3.8	2.32
100			3-9	4	4.6	1.13	97			2-9	4	4.2	1.03
64			2-5	3	2.7	0.74	50	14*	6	2-5	2	2.7	0.77
77		56	3-12	4	3.6	1.63	73			2-7	3	3.2	1.18
100			3-7	4	4.5	1.50	100		10	3-11	6	6.3	1.44
90			2-6	3	3.5	1.03	68			2-6	3	3.0	0.89
76			2-5	3	2.9	0.73	66			1-6	3	3.0	0.98
100	16		3-12	6	5.9	2.34	100			3-11	4	5.6	2.19
100			3-10	4	5.4	1.51	100	12*		4-15	5	5.7	2.24
37			2-7	2	2.6	1.05	21			2-4	2	2.4	1.05

Results

Normal Limits, Sensitivity, and Specificity of the LAVysion Probe Set in NSCLC Imprints

Normal limits for the LAVysion assay for NSCLC imprints were established by determining the frequencies of abnormal cells in imprints of normal tonsillar, bronchial or alveolar specimens. Most cells of normal control specimens displayed two signals for each DNA target (Figure 1A) but a small proportion of cells in the controls had an increased signal number. The average number of signals per cell and standard deviation (SD) for normal and tumor specimens are presented in Table 1.

To determine whether the tumors statistically differed from the control specimens, the normal limit for each DNA probe was set at the mean percentage of abnormal cells in the control specimens that resulted in 100% specificity. Using this definition, upper limits of normal were 4.8% for the 5p15.2 probe, 6.1% for CEP6, 7.3% for 7p12, and 5% for 8q24 and specimens with more than these percentages of cells were determined to be aneusomic. When sensitivity is defined as the percentage of carcinomas with aneusomy for at least one DNA target, the sensitivity of the LAVysion probe is 100% (20 of 20 NSCLC tumors classified as abnormal). Table 2 summarizes the FISH results in each of the NSCLC specimens. None of the tumors exhibited loss of signal and the aneusomies consisted exclusively of increased copy number. In all but one case there were increased signals per cell for two or more DNA targets (Figure 1B).

Considerable intercellular heterogeneity was observed among the tumors, with copy number per cell for each DNA target ranging from 2 to 16 for 5p15.2, 2-12 for centromere 6, 2-15 for 7p12 and 2-21 for 8q24. Modal signal numbers ranged from 2 to 12 (median = 3.5) for

5p15, 2-6 (median = 2) for centromere 6, 2-8 (median = 4) for 7p12, and 2-6 (median = 3) for 8q24. The single tumor showing normal FISH signals was BAC4. Interestingly, gene amplifications represented by a large cluster of signals, usually unscorable due to overlap of the signals, were found for two genes, EGFR (7p12) and MYC (8q24). EGFR was amplified in BAC1 and SCC4 (Figure 1C), and MYC in SCC5. Six tumors (BAC3 and BAC4, SCC3, SCC5, SCC8 and SCC9) were classified as hyperaneusomic for at least one target. In tumors SCC3 and SCC9, the hyperaneusomic cells (14% and 12%, respectively) displayed numerous⁴⁻⁵ split signals for the 8q24 probe, and it is conceivable that these split signals may represent gene amplification as double minutes. Since only interphase analysis was performed in these tumor specimens, no supplementary information is available to confirm or rule out this hypothesis. Frequency of gene amplification and/or hyperaneusomy increased with the clinical stage and was 18% (2/11) in stage I tumors, and 67% (6/9) in stage II-IV tumors ($\chi^2 = 4.84$; 1 DF; $P < 0.05$). Gene amplification and hyperaneusomy were not observed in the control specimens.

The LAVysion FISH Patterns in Sputum Specimens

Sputum samples from 14 individuals were evaluated by the LAVysion assay. Three were collected from patients with SCC and had cytology positive for abnormal cells; 11 had normal cytology and were derived from former smokers without cancer. Conservatively, the FISH pattern was classified as abnormal when gains for at least two targets were present. In the cases with normal cytology, FISH patterns were also normal. In sputum from tumor patients, 11 to 34 abnormally shaped nuclei were selected and

Table 3. Patient Demographics, Pathological, and FISH Results in Bronchial Epithelium of Smoker Patients

Patient	Sex	Smoking status	Pack-years	Lung tumor	Site	Histology at culture site	Average histology score	Highest histology score	Dysplasia index	5p15 (12%)	Cen 6 (8%)	7p12 (12%)	8q24 (9%)
1	M	1	30	No	RB3	1	2.4	6	0.31	0	0	1.1	0
2	F	1	30	No	RUL	2	2.1	5	0.2	12.8	10.6	21.3	12.8
3	F	2	30	No	RB6	5.5	2.6	5	0.29	8.4	4.7	3.7	8.4
					RML	1	2.6	5	0.29	7.1	0	4.8	4.8
4	F	2	34	No	RML	1	1	1	0	0.5	0	3.2	1.1
5	F	2	34.5	No	RUL	2	2.7	5	0.33	6	6	7.2	7.2
6	M	1	35	No	LUDB	1	1.75	6	0.13	2.4	3.6	6	3.6
7	M	2	40	No	RB6	5.5	3.7	5	0.43	0.6	0	0.6	0
8	F	1	40	No	LUL	1	1.3	2	0	0	1.1	2.1	1.1
					RML	2	1.3	2	0	1	1.9	1.9	1
9	F	1	40	No	LB6	5.5	4.7	6	0.83	4	2.6	5.3	4
					RUL	5.5	4.7	6	0.83	12.2	4.1	8.2	12.2
10	M	1	41	No	LB6	4	2.3	5	0.38	0	1	1	2
					LUDB	1	2.3	5	0.38	1.1	4.5	2.3	3.4
11	M	2	45	No	RB6	2	3.4	5	0.67	8.5	5.3	7.5	7.5
12	M	2	47	No	RUL	4	2.3	6	0.29	0	0	0	0
13	F	2	47	No	LUDB	5.6	4.6	6	0.78	0	1.1	0	1.1
					RML	5.6	4.6	6	0.78	1.7	5.2	6.9	6.9
14	M	2	52.5	No	LUL	4	3.45	5	0.72	10.2	10.2	15.3	11.9
					RML	5.5	3.45	5	0.72	31.7	31.7	31.7	31.7
15	F	2	60	No	RML	4	1.6	4	0.13	0	0	0	0
16	M	1	60	No	LB4	1	2	5	0.33	1	1	1	2
					LUDB	1	2	5	0.33	4.6	2.3	5.8	5.8
17	M	2	64	No	RB6	1	1.7	4	0.2	21.2	21.2	21.2	21.2
18	F	1	67.5	No	MC	5.5	1.71	5	0.14	2.6	2.6	1.3	1.3
19	F	1	67.5	No	RB6	N/A	1.1	2	0	12	11.4	12	12.7
20	M	1	70	No	MC	1	1.6	5	0.14	1.9	1.3	2.5	1.9
21	M	1	70	No	RUL	1	1.4	2	0	2.1	3.2	1.1	2.1
22	M	2	70	No	LUDB	4.1	3.9	5	0.85	2.1	1	0	1
					RML	2	3.9	5	0.85	2	3.1	2	2
23	M	2	75	No	LUL	N/A	2	4	0.25	0	0	0.6	0
24	M	2	75	No	LUL	1	2	5	0.29	11.5	11.5	12	11.5
25	M	2	75	No	LB1	2	3.8	5	0.7	2.1	4.3	6.4	2.1
					LUDB	5.5	3.8	5	0.7	2.2	0	3.2	1.1
26	M	1	80	No	LUDB	5.5	3.3	5	0.57	0.9	1.8	0.9	1.8
	M	1	80	No	RML	4	3.3	5	0.57	5.9	4	5.9	4
27	M	2	90	No	LBL	1	2.4	5	0.33	16.5	13.2	13.2	13.2
					LUL	1	2.4	5	0.33	3.8	3.8	9.4	5.7
28	M	1	100	No	LUL	7	7	7	1	1	0	0.5	0.5
29	M	2	100	No	MC	1	3.4	5	0.56	16.7	16.7	17.8	18.9
30	M	1	135	No	RB2	1	1.6	4	0.13	1.2	0.6	0.6	0.6
					LUL	1	1.6	4	0.13	0	0	0	6.1
31	M	1	357.5	No	LUDB	1	1	1	0	0	1.5	2.9	0
32	M	2	65	No	LUL	2	3.5	6	0.2	2.2	3.2	0	3.2
33	M	2	47	No	LUL	5.6	3.5	6	0.5	0	1.1	2.2	0
34	F	2	30	Adenoca	LLL	5.6		8		8.8	11.4	4.4	9
35	F	2	38	Adenoca	RUL/RML	2		8		0	0	1.1	0.5
36	M	2	39	SqCa	RML/RLL	2		8		0.6	0.6	1.9	0.6
37	M	2	45	SqCa	LB6	1		8		8.5	7.6	10.4	9.4
38	M	1	90	SqCa	LLL	2		8		5	1	3	2
39	F	2	105	Carcinoid	RUL	2		8		3.3	1.1	7.6	3.3
40	F	2	135	Adenoca	RUL	2		8		11.1	11.1	9.1	10.1
41	M	1	70	SqCa	RB6	1		8		0	0	1.8	0
42	M	1	51	SqCa	LUL	2		8		0	0	0	0

Frequency of cells with abnormal number of copies per probe are identified per specimen.
Cut-off frequencies were defined adjusting for 100% specificity.
Boldface values represent percentages above the threshold.

scored. Abnormal FISH patterns were seen in 27%, 58%, and 91% of abnormal nuclei in the three cases. Two of these specimens had abnormal FISH results for all four probes, while one had gain only for the 7p12 and 8q24 DNA targets (Figure 1F).

Abnormalities in Cultured Nonmalignant BE from High-Risk Subjects

Findings for nonmalignant cultured BE cells from high-risk patients differed from those of tumor imprints. First,

Table 4. Comparison of Patients with Normal and Abnormal FISH Patterns According to Gender, Smoking Status, Presence of Tumor, and Histology

Parameter	Specimen			Patient		
	FISH pattern		P value (χ^2)	FISH pattern		P value (χ^2)
	Normal	Abnormal		Normal	Abnormal	
Gender			NS			NS
Male	29 (54%)	7 (13%)		22 (53%)	6 (14%)	
Female	13 (24%)	5 (9%)		9 (21%)	5 (12%)	
Smoking status			NS			NS
Former	21 (39%)	3 (5%)		15 (36%)	3 (7%)	
Current	21 (39%)	9 (17%)		16 (38%)	8 (19%)	
Gender and smoking status			<0.05			<0.05
Male former smoker	17 (31%)	0		13 (31%)	0	
Male current smoker	12 (22%)	7 (13%)		9 (21%)	6 (14%)	
Female former smoker	4 (7%)	3 (6%)		2 (5%)	3 (7%)	
Female current smoker	9 (17%)	2 (4%)		7 (17%)	2 (5%)	
Presence of tumor			NS			NS
Yes	6 (11%)	3 (5%)		6 (14%)	3 (7%)	
No	36 (67%)	9 (17%)		26 (60%)	8 (19%)	
Histology at the site			NS			
≤ 2	26 (50%)	7 (13%)				
> 2	15 (29%)	4 (8%)				
Average histology in all biopsied sites			NS			NS
≤ 2	14 (31%)	3 (7%)		11 (33%)	3 (9%)	
> 2	22 (49%)	6 (13%)		14 (42%)	5 (15%)	
Highest histology score			NS			NS
≤ 4	9 (17%)	2 (4%)		7 (17%)	2 (5%)	
> 4	33 (61%)	10 (18%)		24 (57%)	9 (21%)	
Dysplasia index			NS			NS
$< 15\%$	11 (24%)	1 (2%)		9 (27%)	1 (3%)	
$> 15\%$	25 (56%)	8 (18%)		16 (48%)	7 (21%)	

NS, not significant.

the number of cells with increased signals in the control BE samples was 3.2- to six-fold higher than those of the control touch preparations. In every BE control culture, approximately 5% of cells displayed a tetrasomic complement for all targets, probably indicating a tetraploid cell clone. This discrepancy in the baseline frequency of abnormalities between BE cells and tissue imprints is likely due to culture conditions rather than to intrinsic differences between cells sampled. The threshold for definition of abnormal BE specimens was set by ROC analysis in which specificity was adjusted to 100%, on the assumption that abnormal cells found in the controls were due to artifacts during culture. As a result of this analysis, the upper limits of normality were defined as 12% of cells with abnormal signals for 5p15.2, 8% for CEP 6, 12% for 7p12, and 9% for 8q24. The variability among the control cultures for each of the four DNA probes was not significant (one-way analysis of variance; $F = 0.61$, $P = 0.6073$).

FISH patterns of cultured BE from high-risk smokers were frequently abnormal. Some of the observed FISH patterns are illustrated in Figures 1D and 1E. The demographic data on the patients and the frequency of cells with abnormal results for each individual DNA target tested are shown in Table 3. Significantly high frequencies of abnormal cells were found in 12 BE samples: one specimen was aneusomic for only one target, three for two targets, two for three targets, and six for all four targets. These 12 specimens originated from 11 patients,

therefore the frequency of specimens with an abnormal FISH pattern was 12/54 (22%) and the frequency of patients with an abnormal FISH pattern was 11/42 (26%). Specifically, 7 specimens (13%) showed aneusomy for 5p15.2, 10 specimens (19%) for CEP6, 8 specimens (15%) for 7p12, and 12 specimens (22%) for 8q24. Aneusomic cells (gain for chromosome 6, 7 and 8 probes) were present in two biopsies from separate sites of the same patient (P14) suggesting that clonal genetic alterations may occur over a wide area of the bronchial mucosa. Abnormal FISH patterns had no association with concurrent presence of tumor, patient gender, and current smoking status, as summarized in Table 4. However, combining gender and smoking status, abnormalities were more frequent in female former smokers in this small sample. The abnormal FISH pattern was not associated with histology at any level, including the histology score at the analyzed lung site, the average histology score in all biopsies of the patient, the highest score in any biopsied area or the dysplasia index for the multiple sites in each patient.

Discussion

This study documents the feasibility of using the specially designed FISH probe panel, LAVysion, as an assay to detect abnormal cells in NSCLC and premalignant bronchial epithelium. All of the NSCLC tested in this series

exhibited significant numerical chromosomal abnormalities, including hyperaneusomy and amplification for probes mapping to the EGFR and MYC loci. The same probes detected abnormalities in all sputa from patients with proven NSCLC, suggesting that these FISH probes may be useful as a diagnostic tool for accessible specimens such as sputa. Finally, explants of bronchial biopsies from high-risk smokers were abnormal by FISH. These abnormalities may represent markers of premalignant genetic damage that may not be evident by conventional histological examination.

These observations are consistent with prior findings in highly aneuploid lung cancers and in solid tumors in general. The most common chromosomal change observed in NSCLC has been extensive aneusomy (gain of 2 or more chromosomes per cell).^{4,16} Chromosomal copy number and amplification data for lung carcinoma and other tumors are currently available online.¹⁷ Abnormalities have been described in almost every chromosome.¹⁸ Classical cytogenetics,^{19–22} interphase FISH^{5,6} and comparative genomic hybridization^{23,24} show gains of chromosomes 6, 7, and 8 in approximately 50% of the NSCLC. Gain in 5p is also a very common recurrent abnormality in NSCLC,^{21,24–28} suggesting that chromosomes 5, 6, 7, and 8 might be suitable targets for diagnostic FISH probes. This suggestion is confirmed by the present study indicating a high level of sensitivity (100%) for the four-probe LAVysion assay in imprints of NSCLC clinical samples.

An important result from the present study is the detection of cells with abnormal FISH patterns in three sputum samples from lung cancer patients. In all of these samples, abnormalities were observed for at least two chromosomes with chromosomes 7 and 8 the most frequently involved. There were no FISH positive cases among the 11 controls. This preliminary finding indicates that satisfactory FISH results can be obtained from stored samples fixed in the conventional fixative, Saccomanno's fluid. The FISH-positive cases were also positive by conventional sputum cytology and FISH may thus be a confirmatory test.

Aneusomy has previously been found in nonmalignant BE adjacent to lung tumors,^{29,30} in the bronchi of high risk patients without invasive carcinoma^{6,31–33} and in oral epithelium of smokers.³⁴ This suggested that the LAVysion probe set may be useful in detecting genomic abnormalities in epithelia of high-risk smokers. To accomplish this we chose to evaluate monolayers of cultured epithelial cells that were harvested onto coverslips at a minimal time after explantation. This permitted unequivocal counts for each cell with virtually no overlap. Although culturing of cells introduces the possibility of *in vitro* introduction of chromosomal abnormalities, the procedure used here for establishing control values for normal cells was conservative and the proportion of abnormal specimens reported here may underestimate the true level of aneuploidy in the high-risk smoking population.

Using the cut points determined by ROC analysis as described above, we were able to identify significant aneuploidy in cultured BE from 26% of high-risk smokers

including 3 patients with concurrent lung cancer and 8 without current or past lung carcinoma. Significant fractions of aberrant cells were detected in 12 specimens (22%) for 8q24, in 8 specimens (15%) for 7p12, and in 7 specimens (13%) for 5p15.2. In most cases the specimens were abnormal for at least two probes but one case was observed in which a single probe was abnormal, suggesting that the presence of even one abnormal chromosome may be of predictive value. In one case, the same FISH abnormalities were detected at two sites, suggesting expansion of a clonal population to encompass BE from the upper lobe, left lung to middle lobe, right lung, a phenomenon that has been described in the context of a p53-mutant clone in a patient with premalignant bronchial cell squamous dysplasia.³⁵

Progressive genetic changes have been associated with severity of histopathological changes.^{29,36} However, in the present series, no association between the LAVysion FISH patterns and histological diagnosis at the site, histology index, highest histology score or dysplasia index was found. This finding suggests that FISH abnormality may be an independent indicator of carcinogenic progression and possibly an independent risk factor for lung cancer. The predictive power of FISH for future malignancy could not be assessed because of the small sample size and incomplete follow-up of the tested cohort. However, this preliminary study suggests that it will be worthwhile to determine the prognostic value of abnormalities detected in FISH assays as biomarkers for lung cancer risk in a large clinical cohort.

Tobacco consumption is considered the major contributing factor in lung cancer deaths and, in bronchial cells, smokers have had higher frequencies of loss and gain for microsatellite markers³⁷ and p53 mutations³⁸ than non-smokers. In the present study, abnormal FISH patterns were common in both current (30%) and former (13%) smokers. Significant association between abnormal FISH patterns and gender were not detected, although it has also been postulated that women are more susceptible to the effects of carcinogens in tobacco than men.³⁹ However, combining gender and smoking status, a significant difference was found among the groups, with former smoker female subjects displaying higher frequencies of abnormalities.

Multitarget FISH assays have shown higher sensitivity than cytology in detecting abnormal cells in ascitic and pleural effusions of patients with breast⁴⁰ and pancreatic tumors,⁴¹ in urine of patients with bladder cancer,⁸ and in brushings and bronchial biopsied cells from patients with lung cancer.⁴ Here we demonstrate the feasibility of using a FISH assay LAVysion incorporating probes for 5p15, 6p11-q11, 7p12 (EGFR), and 8q24 (MYC) for unambiguously and objectively distinguishing NSCLC tumor cells on imprints and sputa from normal lung and lymphoid cells. This multitarget FISH assay also detected chromosomal abnormalities in bronchial cells of high-risk smokers. Validation of these results in a larger trial could establish multicolor FISH as an adjunct to cytological examination of sputum for early detection of lung cancer and as an intermediate endpoint in chemoprevention trials.

Acknowledgments

We thank Mr. Roger Powell for help with *in situ* cultures and Ms. Kathleen Nelder for assistance with the FISH assays.

References

- Hirsch FR, Franklin WA, Gazdar AF, Bunn Jr PA: Early detection of lung cancer: clinical perspectives of recent advances in biology and radiology. *Clin Cancer Res* 2001, 7:5-22
- Zochbauer-Muller S, Gazdar AF, Minna JD: Molecular pathogenesis of lung cancer. *Annu Rev Physiol* 2002, 64:681-708
- Haruki N, Harano T, Masuda A, Kiyono T, Takahashi T, Tatematsu Y, Shimizu S, Mitsudomi T, Konishi H, Osada H, Fujii Y, Takahashi T: Persistent increase in chromosome instability in lung cancer: possible indirect involvement of p53 inactivation. *Am J Pathol* 2001, 159:1345-1352
- Schenk T, Ackermann J, Brunner C, Schenk P, Zojer N, Roka S, Drach J: Detection of chromosomal aneuploidy by interphase fluorescence in situ hybridization in bronchoscopically gained cells from lung cancer patients. *Chest* 1997, 111:1691-1696
- Taguchi T, Zhou JY, Feder M, Litwin S, Klein-Szanto AJ, Testa JR: Detection of aneuploidy in interphase nuclei from non-small cell lung carcinomas by fluorescence in situ hybridization using chromosome-specific repetitive DNA probes. *Cancer Genet Cytogenet* 1996, 89:120-125
- Zozer N, Dekan G, Ackermann J, Fiegl M, Kaufmann H, Drach J, Huber H: Aneuploidy of chromosome 7 can be detected in invasive lung cancer and associated premalignant lesions of the lung by fluorescence in situ hybridization. *Lung Cancer* 2000, 28:225-235
- Heselmeyer-Haddad K, Chaudhri N, Stoltzfus P, Cheng JC, Wilber K, Morrison L, Auer G, Ried T: Detection of chromosomal aneuploidies and gene copy number changes in fine needle aspirates is a specific, sensitive, and objective genetic test for the diagnosis of breast cancer. *Cancer Res* 2002, 62:2365-2369
- Halling KC, King W, Sokolova IA, Meyer RG, Burkhardt HM, Halling AC, Cheville JC, Sebo TJ, Ramakumar S, Stewart CS, Pankratz S, O'Kane DJ, Seelig SA, Lieber MM, Jenkins RB: A comparison of cytology and fluorescence in situ hybridization for the detection of urothelial carcinoma. *J Urol* 2000, 164:1768-1775
- Varella-Garcia M, Gemmill RM, Rabenhorst SH, Lotto A, Drabkin HA, Archer PA, Franklin WA: Chromosomal duplication accompanies allelic loss in non-small cell lung carcinoma. *Cancer Res* 1998, 58:4701-4707
- Hirsch FR, Prindiville SA, Miller YE, Franklin WA, Dempsey EC, Murphy JR, Bunn Jr PA, Kennedy TC: Fluorescence versus white-light bronchoscopy for detection of preneoplastic lesions: a randomized study. *J Natl Cancer Inst* 2001, 93:1385-1391
- Franklin WA, Folkvord JM, Varella-Garcia M, Kennedy T, Proudfoot S, Cook R, Dempsey EC, Helm K, Bunn PA, Miller YE: Expansion of bronchial epithelial cell populations by in vitro culture of explants from dysplastic and histologically normal sites. *Am J Respir Cell Mol Biol* 1996, 15:297-304
- Travis WD, Colby TV, Corrin B, Shimosato Y, Brambilla E. Histological typing of tumours of lung and pleura. World Health Organization International Classification of Tumours. Edited by Sobin LH. Berlin Heidelberg, New York, Springer-Verlag, 1999
- Soria JC, Moon C, Wang L, Hittelman WN, Jang SJ, Sun SY, Lee JJ, Liu D, Kurie JM, Morice RC, Lee JS, Hong WK, Mao L: Effects of N-(4-hydroxyphenyl)retinamide on hTERT expression in the bronchial epithelium of cigarette smokers. *J Natl Cancer Inst* 2001, 93:1257-1263
- Sokolova IA, Bubendorf L, O'Hare A, Legator MS, Jacobson KK, Grilli BSB, Dalquen P, Halling KC, Tamm M, Seelig SA, Morrison LE: A fluorescence in situ hybridization-based assay for improved detection of lung cancer cells in bronchial washing specimens. *Cancer* 2002, 96:306-315
- McNeil BJ, Hanley JA: Statistical approaches to the analysis of receiver operating characteristic (ROC) curves. *Med Decis Making* 1984, 4:137-150
- Sokolova IA, Halling KC, Jenkins RB, Burkhardt HM, Meyer RG, Seelig SA, King W: The development of a multitarget, multicolor fluorescence in situ hybridization assay for the detection of urothelial carcinoma in urine. *J Mol Diagn* 2000, 2:116-123
- Knuutila S, Autio K, Aalto Y: Online access to CGH data of DNA sequence copy number changes. *Am J Pathol* 2000, 157:689-690
- Balsara BR, Testa JR: Chromosomal imbalances in human lung cancer. *Oncogene* 2002, 21:6877-6883
- Feder M, Siegfried JM, Balshem A, Litwin S, Keller SM, Liu Z, Testa JR: Clinical relevance of chromosome abnormalities in non-small cell lung cancer. *Cancer Genet Cytogenet* 1998, 102:25-31
- Matturri L, Lavezzi AM: Recurrent chromosome alterations in non-small cell lung cancer. *Eur J Histochem* 1994, 38:53-58
- Testa JR, Siegfried JM, Liu Z, Hunt JD, Feder MM, Litwin S, Zhou JY, Taguchi T, Keller SM: Cytogenetic analysis of 63 non-small cell lung carcinomas: recurrent chromosome alterations amid frequent and widespread genomic upheaval. *Genes Chromosomes Cancer* 1994, 11:178-194
- Testa JR, Liu Z, Feder M, Bell DW, Balsara B, Cheng JQ, Taguchi T: Advances in the analysis of chromosome alterations in human lung carcinomas. *Cancer Genet Cytogenet* 1997, 95:20-32
- Bjorkqvist AM, Tammilehto L, Nordling S, Nurminen M, Anttila S, Mattson K, Knuutila S: Comparison of DNA copy number changes in malignant mesothelioma, adenocarcinoma, and large-cell anaplastic carcinoma of the lung. *Br J Cancer* 1998, 77:260-269
- Lu YJ, Dong XY, Shipley J, Zhang RG, Cheng SJ: Chromosome 3 imbalances are the most frequent aberration found in non-small cell lung carcinoma. *Lung Cancer* 1999, 23:61-66
- Balsara BR, Sonoda G, du Manoir S, Siegfried JM, Gabrielson E, Testa JR: Comparative genomic hybridization analysis detects frequent, often high-level, overrepresentation of DNA sequences at 3q, 5p, 7p, and 8q in human non-small cell lung carcinomas. *Cancer Res* 1997, 57:2116-2120
- Johansson M, Dietrich C, Mandahl N, Hambræus G, Johansson L, Clausen PP, Mitelman F, Heim S: Karyotypic characterization of bronchial large cell carcinomas. *Int J Cancer* 1994, 57:463-467
- Ried T, Petersen I, Holtgreve-Grez H, Speicher MR, Schrock E, du Manoir S, Cremer T: Mapping of multiple DNA gains and losses in primary small cell lung carcinomas by comparative genomic hybridization. *Cancer Res* 1994, 54:1801-1806
- Luk C, Tsao MS, Bayani J, Shepherd F, Squire JA: Molecular cytogenetic analysis of non-small cell lung carcinoma by spectral karyotyping and comparative genomic hybridization. *Cancer Genet Cytogenet* 2001, 125:87-99
- Inage Y, Ogata T, Yamamoto T, Akaogi E, Horiguchi H, Kamma H, Satoh Y, Ishikawa S, Onizuka M, Sakakibara Y: Topographical analysis of p53 expression and DNA ploidy in early bronchial squamous cell carcinoma and preneoplastic lesions. *Lung Cancer* 2001, 34:351-361
- Smith AL, Hung J, Walker L, Rogers TE, Vuitch F, Lee E, Gazdar AF: Extensive areas of aneuploidy are present in the respiratory epithelium of lung cancer patients. *Br J Cancer* 1996, 73:203-209
- Sozzi G, Miozzo M, Tagliabue E, Calderone C, Lombardi L, Pilotti S, Pastorino U, Pierotti MA, Della Porta G: Cytogenetic abnormalities and overexpression of receptors for growth factors in normal bronchial epithelium and tumor samples of lung cancer patients. *Cancer Res* 1991, 51:400-404
- Crowell RE, Gilliland FD, Temes RT, Harms HJ, Neft RE, Heaphy E, Auckley DH, Crooks LA, Jordan SW, Samet JM, Lechner JF, Belinsky SA: Detection of trisomy 7 in nonmalignant bronchial epithelium from lung cancer patients and individuals at risk for lung cancer. *Cancer Epidemiol Biomarkers Prev* 1996, 5:631-637
- Hittelman WN: Genetic instability in epithelial tissues at risk for cancer. *Ann NY Acad Sci* 2001, 952:1-12
- Ai H, Barrera JE, Pan Z, Meyers AD, Varella-Garcia M: Identification of individuals at high risk for head and neck carcinogenesis using chromosome aneuploidy detected by fluorescence in situ hybridization. *Mutat Res* 1999, 439:223-232
- Franklin WA, Gazdar AF, Haney J, Wistuba II, La Rosa FG, Kennedy T, Ritchey DM, Miller YE: Widely dispersed p53 mutation in respiratory epithelium: a novel mechanism for field carcinogenesis. *J Clin Invest* 1997, 100:2133-2137
- Wistuba II, Lam S, Behrens C, Virmani AK, Fong KM, LeRiche J, Samet JM, Srivastava S, Minna JD, Gazdar AF: Molecular damage in

- the bronchial epithelium of current and former smokers. *J Natl Cancer Inst* 1997, 89:1366–1373
37. Sanchez-Cespedes M, Decker PA, Doffek KM, Esteller M, Westra WH, Alawi EA, Herman JG, Demeure MJ, Sidransky D, Ahrendt SA: Increased loss of chromosome 9p21 but not p16 inactivation in primary non-small cell lung cancer from smokers. *Cancer Res* 2001, 61:2092–2096
 38. Vahakangas KH, Bennett WP, Castren K, Welsh JA, Khan MA, Blomeke B, Alavanja MC, Harris CC: p53 and K-ras mutations in lung cancers from former and never-smoking women. *Cancer Res* 2001, 61:4350–4356
 39. Siegfried JM: Women and lung cancer: does oestrogen play a role? *Lancet Oncol* 2001, 2:506–513
 40. Zojer N, Fiegl M, Angerler J, Mullauer L, Gsur A, Roka S, Pecherstorfer M, Huber H, Drach J: Interphase fluorescence in situ hybridization improves the detection of malignant cells in effusions from breast cancer patients. *Br J Cancer* 1997, 75:403–407
 41. Zojer N, Fiegl M, Mullauer L, Chott A, Roka S, Ackermann J, Raderer M, Kaufmann H, Reiner A, Huber H, Drach J: Chromosomal imbalances in primary and metastatic pancreatic carcinoma as detected by interphase cytogenetics: basic findings and clinical aspects. *Br J Cancer* 1998, 77:1337–1342