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Molecular Profiling of Computed Tomography Screen-Detected Lung Nodules Shows Multiple Malignant Features

María J. Pajares,[1,6] Isabel Zudaire,[1,7] María D. Lozano,[2] Jackeline Agorreta,[1,6] Gorka Bastarrika,[3] Wenceslao Torre,[4] Ana Remírez,[1] Ruben Pio,[1,8] Javier J. Zulueta,[5] and Luis M. Montuenga[1,6]

[1]Oncology Division, Center for Applied Medical Research; Departments of [2]Pathology, [3]Radiology, [4]Thoracic Surgery, and [5]Pulmonary Medicine, Clínica Universitaria de Navarra; and Departments of [6]Histology and Pathology, [7]Genetics and [8]Biochemistry, Schools of Medicine and Sciences, University of Navarra, Pamplona, Spain

Abstract

Rationale and Purpose: **Low-dose spiral computerized axial tomography (spiral CT) is effective for the detection of small early lung cancers. Although published data seem promising, there has been a significant degree of discussion concerning the potential of overdiagnosis in the context of spiral CT–based screening. The objective of the current study was to analyze the phenotypic and genetic alterations in the small pulmonary malignancies resected after detection in the University of Navarra/International Early Lung Cancer Action Project spiral CT screening trial and to determine whether their malignant molecular features are similar to those of resected lung tumors diagnosed conventionally.**

Experimental Design: **We analyzed 17 biomarkers of lung epithelial malignancy in a series of 11 tumors resected at our institution during the last 4 years (1,004 high-risk individuals screened), using immunohistochemistry and fluorescence *in situ* hybridization (FISH). A parallel series of 11 gender-, stage-, and histology-matched lung cancers diagnosed by other means except screening was used as control.**

Results: **The molecular alterations and the frequency of phenotypic or genetic aberrations were very similar when screen-detected and nonscreen-detected lung cancers were compared. Furthermore, most of the alterations found in the screen-detected cancers from this study were concordant with what has been described previously for stage I-II lung cancer.**

Conclusions: **Small early-stage lung cancers resected after detection in a spiral CT-based screening trial reveal malignant molecular features similar to those found in conventionally diagnosed lung cancers, suggesting that the screen-detected cancers are not overdiagnosed.** (Cancer Epidemiol Biomarkers Prev 2006;15(2):373–80)

Introduction

Lung cancer is responsible for close to 20% of all cancer deaths. In the United States, >160,000 new lung cancers were identified in 2004 (1). Despite improvements in the treatment of advanced disease, the 5-year survival of lung cancer patients is <15%, causing more deaths than breast, prostate, and colon cancers combined (1). Among the main reasons behind the dismal statistics of lung cancer is the lack of effective techniques for early detection. Less than 20% of patients are diagnosed in early stages when surgical intervention is possible. Since the seminal study by Henschke et al. (2) on spiral computed tomography (spiral CT)–based early detection, and as a result of rapid technical refinements, localized small lung cancers are being found more frequently and excitement about the possibility of screening high-risk individuals has grown (3).

Thus far, spiral CT-based lung cancer screening studies have reported high rates of detection of small cancers in early stages, 70% to 90% of the tumors being stage I or II, with an average size of 1.5 cm (2, 4-10). Although these data are very promising, there has been intense discussion concerning the possibility of overdiagnosis. Some authors suggest that CT screening protocols may find a high proportion of indolent cancers that may not cause a significant reduction in the life expectancy of screenees (11, 12). According to this view, these tumors could remain latent and localized for the entire life of an individual. A way to test the existence of overdiagnosis is by analyzing whether the biological traits and the natural history of the resected tumors are identical to cancers diagnosed conventionally. If in the setting of a screening trial, a high proportion of resected lung tumors were not fatally malignant, then the benefits of the screening program would be minimal due to overdiagnosis. To address this question, molecular profiling of the lesions resected within a screening protocol may be very helpful. This analysis will provide a clearer picture of the phenotypic and genetic alterations present in these lesions and an indication of their potential lethality. The aim of this study was to perform biological profiling of the tumors resected within our institutional International Early Lung Cancer Action Project spiral CT screening trial. A number of molecular changes leading to lung cancer have already been described (13). To assess the biological aberrance present in the screen-detected tumors, a panel of biomarkers mostly related to the ''hallmarks of cancer'' (14) were selected. Specifically, markers involved in cell cycle (Ki-67, p16, cyclin D1, cyclin E, p53, and p21), apoptosis (Bcl-2 and Bax), invasion and angiogenesis [matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF)], and mRNA processing [heterogeneous nuclear ribonucleoprotein (hnRNP) A1, hnRNP K, and αCP-4] were analyzed. Additionally, chromosomal imbalances using a set of lung cancer-related probes (15) were investigated. Finally, comparisons between the molecular traits of these tumors with

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Requests for reprints: Luis M. Montuenga, Área de Oncología. Centro para la Investigación Médica Aplicada, University of Navarra, Avenida Pío XII, 55, 31008 Pamplona, Spain. Phone: 34-948194700; Fax: 34-948194714. E-mail: lmontuenga@unav.es

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those of a control group of gender-, stage-, and histology-matched lung cancers diagnosed conventionally (not in the setting of a screening program) were carried out.

Materials and Methods

Population. One thousand and four current and former smokers of at least 40 years of age with no symptoms of lung cancer participated in the screening program. A background questionnaire and written informed consent were obtained. The study protocol was approved by the ethics committee of our center. Eighteen screen-detected lung tumors were diagnosed and resected, 11 of which were available for biomarker studies. Histologic diagnosis and classification of the specimens were defined based on WHO criteria (16). Pathologic staging of the tumors was done according to the international system for staging lung cancer (17). A second series of gender-, stage-, and histology-matched nonscreening diagnosed lung tumors was also studied. These additional patients were diagnosed with lung cancer after presenting with symptoms or because of a casual finding in a diagnostic work-up for other conditions that included either a chest X-ray or a CT.

Immunohistochemistry. For immunohistochemical analysis, 3 μm formalin-fixed, paraffin-embedded sections were dewaxed in xylene and rehydrated through a graded alcohol series. Endogenous peroxidase activity was blocked by placing sections in 3% hydrogen peroxide in water for 10 minutes. Sections were rinsed in deionized water, and, then, when necessary, antigen retrieval was carried out. Tissues were washed in TBS [0.01 mol/L Tris and 0.1 mol/L NaCl (pH 7.36)] and incubated in 5% normal serum for 30 minutes at room temperature to block nonspecific staining. Later, the diluted primary antibody was applied and left overnight at 4°C. Technical conditions of immunohistochemical procedure (type of retrieving buffer and conditions, source of primary antibodies, and dilutions) are indicated in Table 1. Sections were washed in TBS and incubated with monoclonal or polyclonal Envision complex (DAKO, Glostrup, Denmark) for 30 minutes at room temperature. After washing the slides, development of peroxidase with diaminobenzidine was performed. Counterstaining was carried out with hematoxylin or methyl green. Sections were dehydrated through graded alcohol steps and mounted in DPX mounting medium.

Positive and negative controls were included in each experiment. Negative controls were done, leaving out the primary antibody. As positive control, we used tissue previously shown to express the antigen of interest. When possible, internal positive controls were used to interpret the intensity of the staining on the tumor cells.

Immunostaining Evaluation. Two observers (M.J. Pajares and M.D. Lozano) evaluated the extension and intensity of the staining for each antibody in all the samples. Both observers evaluated the samples independently and blinded to the method used for diagnosis. The extension was scored as percentage of positive tumor cells (0-100%) and the intensity of staining was assessed compared with a known internal or external positive control (1+, mild labeling; 2+, moderate; and 3+, intense labeling). Discordant independent reading was resolved by simultaneous review by the two observers.

We chose the cutoff point for each investigated biomarker based on previously published reports. Ki-67 labeling index was defined as high if >20% of the tumor nuclei were positive and low if ≤20% were positive (18, 19). For p16 immunostaining, tumors were considered positive when >10% of the cells showed nuclear staining (20). Cyclin D1, cyclin E, p53, and p21 were considered overexpressed when >5% of the cells showed nuclear immunoreactivity (21-24). A tumor was considered positive for Bcl-2, Bax, and MMP-9 if staining intensity was 2+ or more in at least 20% of the tumor cells (25, 26). Immunostaining scores for VEGF were calculated by the product of the percentage of labeled tumor cells multiplied by the intensity of the staining. The tumors were considered positive when the score was ≥10. The mean staining score was used as the cutoff value to define two groups, one presenting high levels and the other low levels of VEGF expression (27). Tumors were considered positive for hnRNP A1 and K if >50% of the cells showed nuclear immunostaining (28). For αCP-4 immunostaining, tumors were considered positive when >10% of the cells showed cytoplasmic staining (29).

For Ki-67, cyclin D1, cyclin E, p53, and p21, appropriate external positive controls were used. Fibroblast and endothelial cells as well as normal adjacent lung epithelium showing nuclear reactivity were used as positive internal controls for p16. Other internal positive controls were tumor-infiltrating lymphocytes for Bcl-2, normal bronchial epithelium for Bax, and stromal fibroblast and infiltrating macrophages for MMP-9. Smooth muscle cells and hyperplastic type II pneumocytes were used as internal positive controls for VEGF. Normal bronchiolar cells were used as an internal positive control for hnRNP A1 and αCP-4.

Fluorescence *In situ* Hybridization. Interphase FISH analysis was done on 3 μm paraffin-embedded tissue sections. Briefly, slides were deparaffinized and pretreated with 10 mmol/L citrate acid buffer (pH 6) and 0.5 mg/mL pepsin solution. The sections were dehydrated through a series of

Table 1. Dilutions, retrieval conditions, and sources of primary antibodies used for the immunohistochemical assays

Antigens	M/P	Antibody clone	Source	Retrieving buffer	Retrieving conditions (MW)	Dilution
Ki-67	M	MIB-1	DAKO	10 mmol/L Tris/HCl, 1 mmol/L EDTA (pH 9)	20 min	1:80
p16	M	16P07	Neomarkers (Fremont, CA)	0.5 mmol/L EDTA (pH 8)	2 × 5 min	1:1600
Cyclin D1	M	AM29	Zymed (San Francisco, CA)	0.5 mmol/L EDTA (pH 8)	3 × 5 min	1:50
Cyclin E	M	13A3	Novocastra (Newcastle, UK)	MS Unmasker (Microm, France; pH 8)	3 × 5 min	1:10
p53	M	DO-7	Novocastra	10 mmol/L Citrate (pH 7)	20 min	1:200
p21	M	SX118	DAKO	10 mmol/L Tris/HCl, 1 mmol/L EDTA (pH 9)	20 min	1:50
Bcl-2	M	C-2	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)	10 mmol/L Citrate (pH 6)	2 × 15 min	1:100
Bax	M	B-9	Santa Cruz Biotechnology	10 mmol/L Citrate (pH 6)	2 × 15 min	1:100
MMP-9	P	—	Neomarkers	—	—	1:100
VEGF	P	—	Santa Cruz Biotechnology	10 mmol/L Citrate (pH 6)	2 × 15 min	1:250
hnRNP A1	M	4B10	G. Dreyfuss (Philadelphia, PA)	—	—	1:6000
hnRNP K	P	—	University of Navarra	10 mmol/L Citrate (pH 6)	2 × 15 min	1:100
αCP-4	P	—	University of Navarra	—	—	1:50

Abbreviations: M, mouse monoclonal antibody; P, rabbit polyclonal antibody; MW, microwave heating.

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Table 2. Clinicopathologic features of the 22 non–small cell lung cancer patients

Patient no.	Detection method	TNM	Histology	Size (cm)	Years	Pack-years	Smoking status*
1	SDT: CT scan	$T_1N_0M_0$	ADC	1.6	58	45	Smoker
2	SDT: CT scan	$T_1N_0M_0$	SQC	1.8	49	20	Smoker
3	SDT: CT scan	$T_1N_0M_0$	SQC	2.0	58	75	Ex-smoker
4	SDT: CT scan	$T_2N_0M_0$	ADC	1.7	62	92	Ex-smoker
5	SDT: CT scan	$T_1N_0M_0$	SQC	0.6	60	102.5	Ex-smoker
6	SDT: CT scan	$T_1N_0M_0$	SQC	0.9	65	60	Smoker
7	SDT: CT scan	$T_1N_0M_0$	ADC	1.1	54	62.5	Smoker
8	SDT: CT scan	$T_1N_0M_0$	ADC	1.2	55	63	Smoker
9	SDT: CT scan	$T_1N_0M_0$	SQC	1.3	56	30	Smoker
10	SDT: CT scan	$T_1N_0M_0$	ADC	0.8	65	24	Ex-smoker
11	SDT: CT scan	$T_1N_0M_0$	ADC	2.5	82	82.5	Ex-smoker
12	NSDT: symptoms	$T_1N_0M_0$	ADC	1.8	81	7	Ex-smoker
13	NSDT: symptoms	$T_1N_0M_0$	SQC	2.0	70	96	Smoker
14	NSDT: CT scan	$T_1N_0M_0$	SQC	2.5	72	13.5	Smoker
15	NSDT: NA†	$T_2N_0M_0$	ADC	3.8	67	132	Smoker
16	NSDT: CXR	$T_1N_0M_0$	SQC	1.3	63	100	Ex-smoker
17	NSDT: CXR	$T_1N_0M_0$	SQC	2.5	75	0	Nonsmoker
18	NSDT: CT scan	$T_1N_0M_0$	ADC	1.0	59	50	Smoker
19	NSDT: NA	$T_1N_0M_0$	ADC	2.0	42	NA	Smoker
20	NSDT: CXR	$T_1N_0M_0$	SQC	2.5	64	80	Smoker
21	NSDT: CT scan	$T_1N_0M_0$	ADC	1.7	72	30	Ex-smoker
22	NSDT: CXR	$T_1N_0M_0$	ADC	1.0	58	18	Smoker

NOTE: All the patients in this study were male.
Abbreviations: SDT, screen detected tumors; NSDT, nonscreen-detected tumors; CXR, chest X-ray; SQC, squamous cell carcinoma; ADC, adenocarcinoma; NA, Not available
*at surgery.
†Initial diagnosis was carried out in a different institution.

ethanol washes and codenatured with LAVysion set probe at 70°C for 10 minutes. Posthybridization washes were done for 2 minutes at 72°C in 2× SSC. Later, tissues were counterstained with 4′,6-diamidino-2-phenylindole. The samples were evaluated on a Zeiss Axioplan2 fluorescent microscope (Zeiss, Welwyn Garden City, United Kingdom) and multifocus images were acquired for all probes using different focal planes with the ISIS software (Metasystems, Altlussheim, Germany).

FISH Data Evaluation. Evaluation of FISH signals was done by counting 60 intact and nonoverlapping nuclei per slide belonging to several different areas. To establish the cutoffs for abnormality, four normal-appearing bronchiolar epithelium sections were also analyzed. The average number of signals per cell and the percentage of abnormal cells were scored from the three groups (normal lung tissue, screen-detected tumors, and nonscreen-detected tumors). Statistical significant cutoffs were calculated in the normal-appearing lung tissues as the average number of signals per cell plus thrice the SD for each probe scored.

Statistical Analysis. To determine whether the molecular profile of the screen-detected tumors shared similarity with nonscreen-detected tumors, a Fisher's exact test was done to compare the results of each immunohistochemical abnormality. The Kruskall-Wallis test was used to compare the FISH profile within the three groups. Independent groups were compared using the Mann-Whitney *U* test. Data analysis was done using SPSS software version 11.0.

Results

Pathologic Findings. All resected screen-detected nodules were diagnosed as malignant lung tumors. No resected nodule was diagnosed as benign. From a histologic point of view, five squamous cell carcinomas and six adenocarcinomas were diagnosed among the screen-detected tumors. All the adenocarcinomas were moderately differentiated tumors, showing epithelial malignant cells with some degree of glandular differentiation. The squamous cell tumors were composed of sheets and cords of epithelial atypical cells without glandular

Table 3. Results of the immunohistochemical analysis

	Screen-detected tumors											Nonscreen-detected tumors										
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Ki-67	L	H	H	H	H	H	H	L	H	H	L	L	H	H	H	H	H	L	H	H	L	L
p16	+	+	−	−	−	−	+	+	−	−	+	+	−	−	+	+	−	+	−	−	−	+
Cyclin D1	−	−	+	+	+	−	−	+	−	−	+	+	+	+	−	−	+	+	−	−	+	−
Cyclin E	−	+	+	−	+	+	+	+	+	−	−	+	−	+	−	+	+	+	+	−	−	−
p53	−	+	+	−	−	+	−	−	+	−	−	+	−	+	+	−	+	−	−	−	−	−
p21	−	+	−	+	+	+	+	−	+	−	−	+	+	−	+	+	+	−	−	+	−	−
Bcl-2	−	+	+	−	−	−	+	+	+	−	−	+	−	+	+	−	+	−	−	+	−	−
Bax	−	+	−	+	−	+	+	+	+	−	+	−	+	−	+	+	+	+	−	+	−	−
MMP-9	+	+	+	−	+	+	−	−	+	+	+	−	−	+	+	+	+	+	−	+	+	+
VEGF	L	L	H	H	L	H	L	L	L	L	H	H	L	L	H	L	H	L	L	H	L	L
hnRNP A1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
hnRNP K	+	+	−	+	−	+	−	−	+	+	+	+	+	−	+	+	+	+	−	−	−	+
αCP-4	−	−	−	−	+	−	−	+	+	+	+	+	−	+	−	−	+	−	−	−	−	−

Abbreviations: H, high expression; L, low expression.

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differentiation. One of the screen-detected squamous carcinomas presented a significant lymphoid infiltrate and was classified as a lymphoepithelioma like carcinoma (patient SDT3). According to the international system for staging of lung cancer, 10 tumors were staged as IA and one tumor was staged as IB. The control series of nonscreen-diagnosed lung tumors was matched by histology and stage with the series of screen-detected tumors. Clinical and pathologic

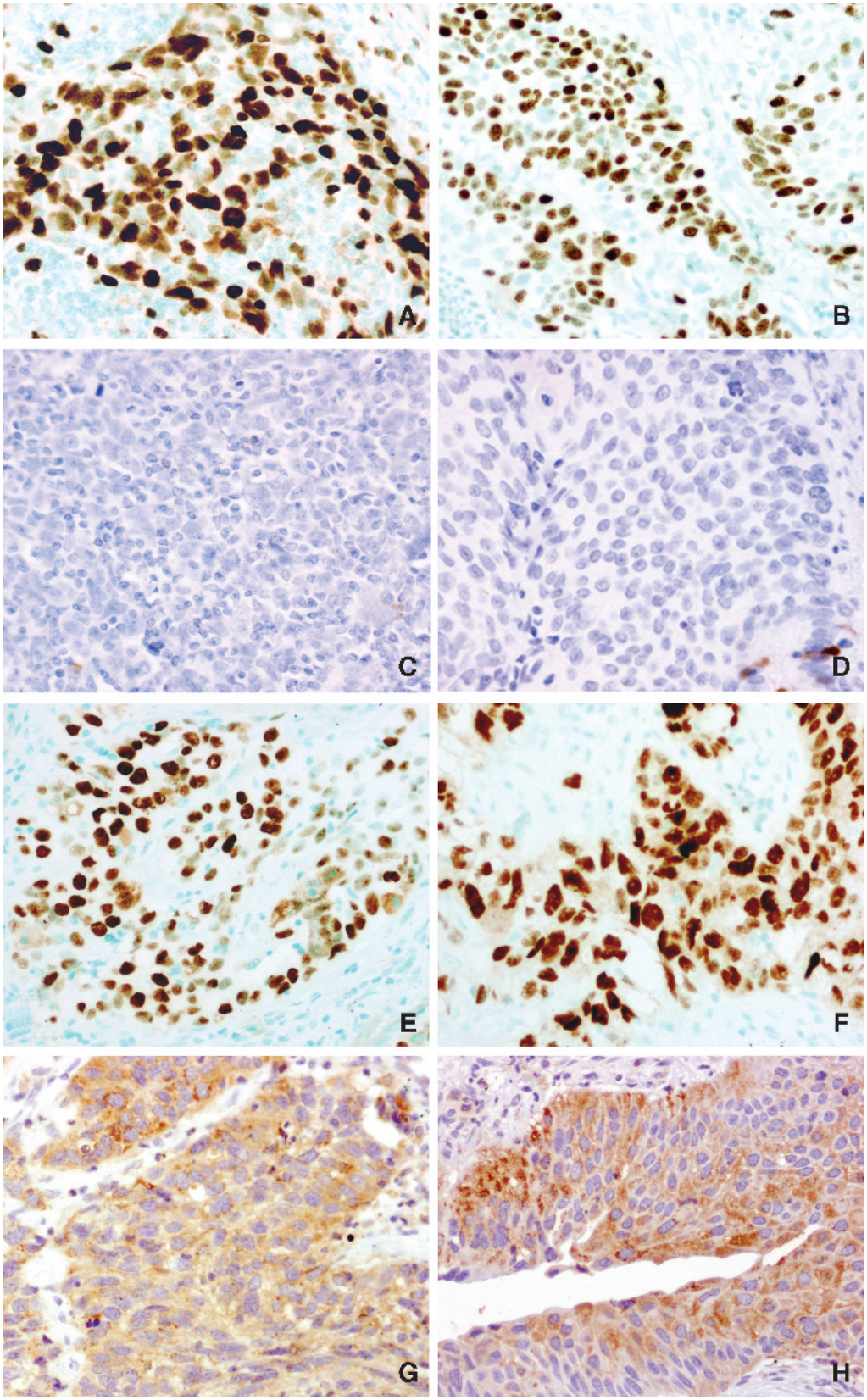

Figure 1. Examples of alteration patterns of proteins in screen-detected lung tumors (**A, C, E**, and **G**) compared with nonscreen-detected ones (**B, D, F**, and **H**). **A** and **B**, Ki-67 expression. **C** and **D**, p16 loss of expression. **E** and **F**, p53 protein accumulation. **G** and **H**, VEGF expression.

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features of both series of patients are described in Table 2. There were no statistically significant differences in age, tumor size, and pack-years between the two groups.

Immunohistochemical Assays. Table 3 summarizes the results on the 13 immunohistochemical markers in the two series of lung tumors investigated.

Cell Cycle–Related Proteins. Comparing screen-detected (n = 11) with nonscreen-detected (n = 11) lung cancers (Fig. 1), the following abnormalities were found: eight screen-detected and seven conventionally detected tumors presented high levels of Ki-67; expression of p16 protein was lost in six tumors in each group; cyclin D1 was altered in five and six tumors, respectively; and cyclin E was overexpressed in seven and six tumors, respectively. Most of the cancers (10 in screen-detected and 11 in nonscreen-detected tumors) had alterations in at least one protein of the Rb pathway. Four tumors out of 11 in each group of cancers were positive for p53 accumulation, whereas five cancers in each group had lost expression of p21. At least one of the p53-p21 pathway proteins analyzed was altered in eight cases in both the screen-detected and nonscreen-detected cancers. Normal lung epithelium, both alveolar and bronchiolar, was negative for all the studied cell cycle–related proteins.

Markers of Apoptosis. Bcl-2 was positive in 5 of 11 tumors in both screen-detected and nonscreen-detected tumors. Immunohistochemical labeling for Bax protein was found in seven screen-detected tumors and in six conventionally detected tumors. Normal bronchial epithelium was positive for Bcl-2 and Bax in all the cases studied.

Markers of Invasion and Angiogenesis. Eight of 11 tumors were positive for MMP-9 in both screen-detected and nonscreen-detected tumors. VEGF was highly expressed in four tumors in each group. Normal bronchial epithelial cells expressed high levels of VEGF and MMP-9.

RNA-Binding Proteins. All the lung cancers from both study groups overexpressed at least one of the RNA-binding proteins investigated. All the tumors from both groups showed hnRNP A1 expression in most of the cells. Intensity of immunostaining was higher in the tumor cells than in the normal bronchiolar epithelial cells. In seven cancers from each group, hnRNP K expression was found in neoplastic cells but not in normal cells. Expression of αCP-4 was observed in the cytoplasm of normal bronchiolar or bronchial cells and was lost in six and eight cancers from the screen-detected and the nonscreen-detected group, respectively.

In summary, all the tumors had abnormalities in at least three of the four investigated pathways (cell cycle, apoptosis, invasion and metastasis, and RNA processing) and in at least 7 of the 13 lung cancer-related proteins analyzed. Statistical analysis comparing the frequencies of aberrant expression showed that there were no significant differences for any of the biomarkers studied by immunohistochemistry between screen-diagnosed and nonscreen-diagnosed tumors ($P > 0.05$, Fisher's exact test).

FISH Analysis. Successful hybridization was observed in 11 (100%) screen-detected tumors and 10 (90.9%) nonscreen-diagnosed tumors. Control tumor number 19 was excluded from the FISH analysis because of the suboptimal FISH signal intensity. Three screen-detected tumors and one control matched case also showed suboptimal results for *epidermal growth factor receptor* (*EGFR*) probe due to weakness of the FISH signals. The average number of signals per cell and the percentage of abnormal cells are summarized in Table 4. The results of the FISH analysis of the normal-appearing lung tissues established the cutoff values of 2.2 for the 5p15.2 probe, 1.9 for *EGFR*, 2.1 for *MYC*, and 2.3 for the centromeric probe of chromosome 6. With the exception of two screen-detected tumors, which had a normal number of copies of chromosome 6, and one conventionally diagnosed tumor with normal *EGFR* and CEP6 signals, all the other tumors analyzed showed an abnormal pattern of signals for all four probes (Fig. 2).

Considerable intercellular heterogeneity was observed among the tumors, with copy number per cell in screen-detected tumors ranging from 1 to 20 for 5p15.2, 1 to 12 for centromere 6, 1 to 10 for *EGFR*, and 1 to 18 for *MYC*. Conventionally detected tumors showed a similar range of heterogeneity in the signals.

The Kruskal-Wallis test detected significant differences in the FISH quantitative results between normal tissue and the

Table 4. Number of FISH signals for each probe and frequencies of abnormal cells in the tumors

	5p15 (1.9;0.1)(2.2)*				*EGFR* (1.7;0.05)(1.9)				*MYC* (1.8;0.09)(2.1)				CEP6 (1.8;0.19)(2.3)			
	Ab	Average	SD	Range	Ab	Average	SD	Range	Ab	Average	SD	Range	Ab	Average	SD	Range
SDT 1	31	2.5	1.03	1-6		SO			38	2.9	1.71	1-9	14	2.1	0.49	1-4
SDT 2	50	2.9	1.66	1-9	33	3.4	1.89	1-9	33	2.7	1.56	1-9	54	2.8	1.42	1-9
SDT 3	100	7.1	3.92	3-20	90	4.9	2.34	2-10	95	6.2	3.45	2-18	100	5.4	2.51	3-12
SDT 4	93	5.4	1.89	2-10		SO			100	5.5	1.4	3-8	96	4.2	1.02	2-6
SDT 5	65	3.3	1.89	1-12	47	3.1	1.89	1-10	45	2.9	1.90	1-14	55	2.9	1.26	1-8
SDT 6	93	5.2	2.35	1-14	32	2.3	1.26	1-6	70	3.7	1.85	1-10	62	3.2	1.89	1-7
SDT 7	81	3.6	1.23	1-6	37	2.5	1.02	1-5	37	2.9	1.30	1-7	37	3.0	1.35	1-7
SDT 8	98	7.3	3.29	2-18	88	3.7	1.35	2-9	82	4.1	1.90	1-9	86	3.3	1.36	1-8
SDT 9	88	3.8	1.29	2-7		SO			68	2.8	0.98	1-5	3	1.5	0.62	1-4
SDT 10	39	2.4	0.95	1-5	7	1.8	0.55	1-3	16	2.1	0.79	1-5	26	2.1	0.83	1-5
SDT 11	90	4.3	1.66	1-10	70	3.0	0.99	1-6	85	3.9	1.77	1-10	13	1.9	0.93	1-6
NSDT 12	65	3.1	0.89	2-4	25	2.0	0.76	1-3	94	8.6	4.30	2-12	28	2.4	0.97	1-4
NSDT 13	80	3.4	1.12	1-6		SO			86	3.6	1.32	2-7	55	2.8	1.03	1-6
NSDT 14	97	4.7	1.49	2-9	43	2.6	0.82	1-5	95	5.5	2.21	2-11	75	3.4	1.26	1-7
NSDT 15	97	6.2	2.35	2-15	78	3.1	1.01	1-7	97	5.7	1.93	2-10	60	2.9	1.05	15
NSDT 16	73	3.3	1.23	1-7	10	1.9	0.53	1-3	63	2.8	1.00	1-6	7	1.9	0.53	1-4
NSDT 17	100	5.9	1.99	3-11	55	2.7	1.00	1-5	68	3.2	1.19	1-7	58	2.9	1.30	1-7
NSDT 18	97	4.6	1.64	1-9	49	2.7	1.13	1-6	97	4.6	1.51	2-10	75	3.3	1.13	2-7
NSDT 19		SO				SO				SO				SO		
NSDT 20	90	5.0	2.07	2-12	50	2.6	1.32	1-9	58	3.1	1.67	1-9	45	2.5	1.35	1-6
NSDT 21	12	1.9	0.66	1-4	13	2.1	0.49	1-4	3	1.9	0.50	1-4	8.3	1.9	0.65	1-4
NSDT 22	100	7.3	1.99	4-12	71	2.9	0.93	1-5	73	3.1	1.25	1-9	84	3.7	1.59	1-9

Abbreviations: Ab, percentage of abnormal cells per probe; SO, suboptimal FISH signal intensity.
*(Average; SD)(cutoff) values.

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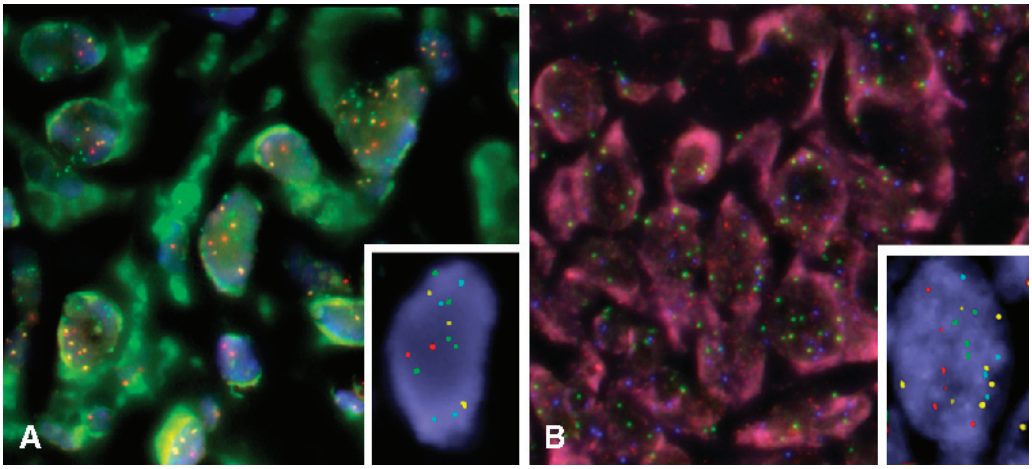

Figure 2. Examples of FISH analysis. Sequence from chromosome 5p15 is labeled with SpectrumGreen, centromere 6 probe is labeled with SpectrumAqua (*blue dots*), *EGFR* probe is labeled with SpectrumRed, and *CMYC* probe is labeled with SpectrumGold (*yellow dots*). **A,** Screen-detected lung tumor (case SDT 5). **B,** Nonscreen-detected tumor (case NSDT 20).

tumor groups for all four probes ($P = 0.010$ for 5p15, $P = 0.006$ for *EGFR*, $P = 0.008$ for *MYC*, and $P = 0.041$ for CEP6). Using the Mann-Whitney U test, significant differences between normal lung tissue and screen-detected tumors, and between normal lung tissue and nonscreen-detected tumors, were found. No statistically significant difference in any of the probes was found between screen- and nonscreen-detected tumors (Table 5).

Discussion

Spiral CT can detect lung cancer as small as several millimeters in diameter and at very early stages (2, 10, 30). However, it has been suggested that a proportion of these early diagnosed lung cancer may be overdiagnosed cases (benign nodules that are treated as malignant or slow-growing tumors that would not cause the demise of the patient). The present work summarizes data on the phenotypic and genetic alterations present in the tumors resected in the setting of a spiral CT screening trial, and suggests that these lesions can be considered fully neoplastic according to their biomarker profile. Although the size of the series investigated is limited, it is obtained from a screened cohort of >1,000 individuals, thus providing relevant information on the malignant features from a molecular point of view of tumors found in a screening setting. These data support the view that screen-detected tumors by spiral CT share the same molecular profile as conventionally detected lung cancers of the same clinicopathologic stage.

A wealth of information has accumulated over the last decade on the alterations in the expression of several genes in lung cancer cells compared with normal lung epithelium. The current view of lung carcinogenesis is a multistep process starting as a normal epithelium, which progressively accumulates molecular abnormalities from the preneoplastic to the neoplastic stages (13). To evaluate the molecular abnormalities in screen-detected lung cancers resected at our institution, 17 biomarkers frequently involved in lung cancer were analyzed: 13 phenotypic molecular alterations and 4 genetic aberrations. Among the former, phenotypic alterations analyzed included the expression of proteins related to cell cycle, apoptosis, invasion, angiogenesis, and mRNA metabolism. Changes in cell cycle–related proteins, especially Ki-67 overexpression, dysregulation of the Rb-cyclin D1-p16 pathway and p53-p21^{WAF1} alterations, are very common biomarkers for lung cancer (31). In fact, several reports have shown that 90% of lung tumors have at least one of the Rb pathway proteins altered (32-34). In our series of early lung tumors, we have evaluated three Rb pathway proteins and, in agreement with other reports, we have observed modifications of at least one protein of this pathway in 80% to 90% of the tumors both in the screening and the nonscreening groups. Similarly, most of these tumors showed alterations in the p53-p21 pathway. In relation to apoptosis, our data showed alterations in the expression of at least one of the two markers studied, Bcl-2 and Bax, in around 75% of the cases in both groups. Other key mechanisms in lung cancer development and progression include the dysregulation of angiogenesis and invasion. VEGF, an angiogenic factor (27, 35), and MMP-9, an invasion marker especially active in extracellular matrix degradation (25), are overexpressed in most of the tumors analyzed. The relevance of modifications in alternative mRNA splicing has been recently stressed (36), and in this regard, the hnRNP family is involved in the regulation of transcription, mRNA metabolism, and translation. Overexpression of hnRNP A2/B1, a member of the hnRNP family, has been reported as a potential biomarker for early detection of lung cancer (37),

Table 5. Comparison of FISH results between normal lung tissues, screen–detected and nonscreen–detected tumors

	5p15 (2.2)*				*EGFR* (1.9)				*MYC* (2.1)				CEP6 (2.3)			
	Average	SD	$P^{\dagger}$	$P^{\ddagger}$	Average	SD	$P^{\dagger}$	$P^{\ddagger}$	Average	SD	$P^{\dagger}$	$P^{\ddagger}$	Average	SD	$P^{\dagger}$	$P^{\ddagger}$
Normal	1.9				1.7				1.8				1.8			
SDT	4.3	1.71	0.001	0.725	3.1	0.96	0.004	0.200	3.6	1.26	0.001	0.468	2.9	1.11	0.040	0.918
NSDT	4.5	1.64	0.004		2.5	0.41	0.003		4.2	1.96	0.004		2.8	0.61	0.008	

*Cutoff values.
†P values comparing normal and screen-detected tumors, and normal and nonscreen-detected tumors are calculated using the Mann-Whitney U test.
‡P values comparing the two groups of tumors are calculated using the Mann-Whitney U test.

whereas other members of the same family have been shown to be abnormally expressed in lung cancer biopsies and cell lines (28, 29). In the present work, immunohistochemical analysis showed an alteration of the RNA processing mechanisms in both types of tumors studied. Finally, the analysis of genetic alterations by FISH using the LAVysion set also shows a close similarity between the two groups of tumors studied. This panel of probes has been shown to be highly sensitive and specific for lung cancer (15, 38). In this series, all but three tumors showed an aberrant genetic profile for all four probes. Although multicolor FISH analysis is a laborious technique in particular on paraffin-embedded tissues, these results suggest that it could become a very useful tool for the diagnosis of early solid lung tumors. In summary, all the molecular markers studied are similarly altered in screen-detected tumors and in stage I cancers diagnosed outside a screening program.

Bianchi et al. (39) have recently reported a cDNA array analysis of screen-detected and nonscreen-detected tumors. Their results show that the two groups of tumors hardly differ in gene expression profiles. In the present work, the focus was put on a panel of markers that have already been related to lung cancer. In agreement with the aforementioned cDNA study, the almost identical percentages of molecular alterations reported herein support the hypothesis that lung cancer detected in a screening program has a similar malignant potential than conventionally, nonscreen-detected cancer. Furthermore, all the data from both series are concordant with the published literature on resectable non–small cell lung cancer in relation to cell cycle–related proteins (18, 21, 23, 24, 33, 40-42), apoptosis regulators (26, 43, 44), invasive and angiogenic markers (26, 27), RNA processing molecules (28, 29), and genetic aberrations (15, 38).

Results found in screen-detected tumors also fit with previously published reports on differences in the expression of biomarkers according to tumor histology. Thus, high levels of Ki-67 (18, 19, 40), loss of p16 (21), accumulation of p53 (45, 46), cyclin E overexpression (40), and Bcl-2 expression (26, 43, 44, 47) were observed more commonly in squamous cell carcinomas than in adenocarcinomas. However, p21 loss was observed more frequently in adenocarcinomas than in squamous cell carcinomas. In genetic analysis by FISH, there were no significant differences between both histologic types, showing that this multi-FISH assay is a useful tool for detection of both types of tumors.

In summary, with respect to protein and genetic profiles, screen-detected lung cancers showed profound molecular alterations that did not differ significantly from conventionally detected tumors. This provides strong support to the hypothesis that spiral CT–detected tumors in the setting of a screening program are bona fide aggressive malignancies. The number of altered protein markers (at least 7 of 13) and genetic markers (at least 3 of 4) found in all of the cancers studied, as well as the fact that 100% of the cases had at least three of four key molecular pathways altered, suggests that none of them was a mere benign hyperproliferative noninvasive disease with minor clinical adverse consequences. Rather, in screen-detected early tumors, most of the biological hallmarks of aggressive cancer are already present.

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