Overexpression of hnRNPA2/B1 in Bronchoscopic Specimens: A Potential Early Detection Marker in Lung Cancer

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Abstract. *Background: Overexpression of heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 has recently been suggested to be a promising marker for early detection of lung cancer. The aim of this study was to determine the utility of its detection in bronchoscopic specimens. Patients and Methods: Brushing and biopsy specimens were obtained from 61 patients suspected of having lung cancer, as well as from 30 healthy subjects (controls), who underwent bronchoscopy. hnRNPA2/B1 expression levels were evaluated by immunoblotting. Results: Specificity of hnRNP A2/B1 overexpression was 75.9% in brushing and 78.3% in biopsy. Sensitivity in non-small cell lung cancer was 84.8% in brushing and 80.8% in biopsies, while in small cell lung cancer it was 66.7 % and 75% , respectively. Overexpression of hnRNPA2/B1 was also detected in bronchoscopic specimens of nine patients initially undiagnosed. The follow-up of these patients 2 years later showed that seven of them had developed lung cancer. Conclusion: Overexpression of hnRNPA2/B1 was significantly higher in patients suffering from lung cancer and may be useful in the early detection of lung cancer.*

Lung cancer is currently the leading cause of cancer deaths in both sexes in the developed world. Thirty-one percent of cancer deaths in men are attributable to lung cancer, while they are expected to account for about twenty-five percent of all female cancer deaths, surpassing those owing to breast cancer (1). Endobronchial biopsy and brushing are the key diagnostic approach in cases of suspected lung cancer. Nevertheless, conventional cytology is often inconclusive and further invasive procedures are required (2). Therefore, more sensitive diagnostic methods are needed.

The prognosis for patients with lung cancer depends primarily on the stage of the tumor at the time of clinical diagnosis. Early-stage tumors can be curable by surgical resection and early diagnosis of lung cancer is obviously the key to longer-term survival (1). It is, thus, important to find new methods of detecting lung cancer in early stages for a favourable prognosis.

No screening examinations have been established for the diagnosis of lung cancer when it is still in presymptomatic stage. Chest X-ray and sputum cytology are commonly used but these methods have not been proven to be sensitive adequately (3). Use of low-dose spiral computed tomography in screening is currently being studied as a promising screening tool. Molecular markers can be detected in early stages of lung carcinogenesis and include mutations of *p53*, loss of p16 expression, expression of telomerase and loss of heterozygosity of 3p, 9p or 17p. However, currently, no biomarker has been validated for early detection of lung cancer.

hnRNPA2 and its alternatively spliced variant B1, referred to as hnRNPA2/B1, belong to a family of RNA-binding proteins involved in several cellular functions, primarily in regulating gene expression both at the transcriptional and translational level as well as in apoptosis (4, 5). The overexpression of hnRNPA2/B1 seems to indicate neoplastic transformation and may play a role in lung carcinogenesis (6). According to published data, hnRNPA2/B1 may be a useful tool in the early diagnosis of lung cancer (7, 8). Overexpression of hnRNPA2/B1 has been detected in all histological subtypes of lung cancer (9). Recent evidence suggests that hnRNPB1 is more selectively overexpressed than A2 and is detected in several tumors, including lung carcinomas (10, 11), oral squamous carcinoma (12), oral leukoplakia (13) and oesophageal squamous carcinoma (14).

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Overexpression of hnRNPA2/B1 has mainly been studied by immunohistochemistry in sputum (15) and in surgically removed tissue (6). There are limited data about endobronchial biopsy in chronic smokers (9), bronchial lavage (2, 7, 16), and peripheral blood (16, 17, 18). There are no published data concerning hnRNPA2/B1 levels in brushing taken from the same patient. In this study, we used immunoblotting to assess the expression level of the protein in bronchoscopic (brushing and biopsy) specimens of patients with high clinical probability of having lung cancer, as well as in controls, in order to estimate its clinical usefulness of this approach for the early detection of lung carcinogenesis.

Patients and methods

Study population. Ninety-one individuals (61 patients and 30 controls) were enrolled in this study. They were eligible regardless of age, gender, background, Karnofsky score or smoking status and all of them gave written consent. Sixty-one of them had high clinical probability of having lung cancer and underwent bronchoscopy. In 95% of patients, brushing samples were obtained from the site of the abnormal epithelium. In 52% of them, brushing samples were also taken from the normal appearing epithelium of the other lung. Biopsies were obtained from 61% of patients from the site of the lesion (a); in 1/3 of patients, biopsies were also taken from adjacent normal appearing epithelium (b) and normal appearing epithelium of the other lung (c). Both brushing and biopsy samples were obtained in 62.3% of cases. Additional bronchoscopic specimens were also sent for cytological or histological examination in order to make a diagnosis. Thirty subjects with no clinical probability for lung cancer and normal chest X-ray, not suffering from any other disease of the lung, underwent bronchoscopy for a different reason (19 pts presented with minor haemoptysis and 11 complained of unexplained cough) and were considered controls. Brushing samples and biopsy from a random location were taken. Both specimens were obtained in 22 patients; they underwent histological and cytological examination and showed no abnormalities.

In order to evaluate our methodology, surgically removed tissue was obtained from 15 individuals: 10 lung cancer patients (8 males, 2 females) who underwent lobectomy or lung resection and 5 patients (4 males, 1 female) without lung cancer who underwent surgery for a different reason (*i.e.* pneumothorax). Biopsies obtained from healthy individuals were also included in the study to determine the baseline levels of hnRNPA2/B1 in relation to cellular actin. Actin and hnRNPA2/B1 protein levels were compared between surgically removed tissue obtained from patients suffering from lung cancer and surgically removed tissue obtained from a random location in patients without cancer. Brushing samples were stored in normal saline at –80˚C without previous handling. Biopsies and surgically removed tissue were quick-frozen in liquid nitrogen and stored at –80˚C until processing. The histological types of lung cancer were determined according to the histological typing of lung and pleural tumors, WHO (19). At the time of the diagnosis, the patient's stage was determined using the Tumor-Node-Metastasis system (20, 21).

Cell culture. Human non-small cell lung cancer cell line A549 was maintained in monolayer culture at 37˚C in RPMI supplemented with 10% fetal calf serum.

Antibodies. Rabbit polyclonal serum (α-A2/B1) was raised against purified rat A2 protein excised from 2D gel. Anti-β-actin monoclonal antibody was purchased from Chemicon International to detect β-actin.

Preparation of extracts for immunoblotting. Collected A549 cells were lysed in lysis buffer (9.5 M urea, 2% Nonidet P-40, 5% β-mercaptoethanol 2% ampholynes) at room temperature. The lysates were clarified at 4000 xg for 10 min and supernatants were obtained as total cellular protein.

Tissue samples were mechanically homogenized in lysis buffer, sonicated (5×10 seconds) and centrifuged. Total protein was trichloroacetic acid (TCA) precipitated from the supernatants.

Brushing samples was centrifuged after thawing in order to pellet cells. The supernatant was kept. The pellet was dissolved in lysis buffer and disrupted by vortexing. Total protein in the cell pellet, as well as in the supernatant was collected by TCA precipitation and combined together. Protein concentration was measured by Bradford Assay (Bio-Rad Laboratories).

Immunoblotting. Western blot is an advanced method in molecular biology that allows the identification of a specific protein in a complex protein mixture. It consists mainly of three stages: the separation of proteins by electrophoresis according to their shape and size, their transfer onto a membrane while retaining their relative position and the detection of a certain protein using specific antibodies. Proteins (25-100 μg) were resolved by a 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis, followed by elecrotransfer to a nitrocellulose membrane that was stained with Ponceaus before probing with antibodies. Membranes were blocked in Zeller solution with 5% skim milk (10 mM Tris-HCL, pH 7.4, 0.5% Tween 20 and 0.1% Triton® X-100) and then they were probed with the desired polyclonal or monoclonal antibody diluted in Zeller overnight at 40˚C, washed in Zeller and incubated with a second antibody (horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin). Antigen/antibody complexes were detected using the Enhanced Chemiluminescence (ECL) system. All samples were coded and the intensity of the hnRNPA2/B1 bands was evaluated and scored by a single-blinded observer in comparison to actin marker bands (of pre-determined increasing concentrations) run on parallel gels. All hnRNPA2/B1 values were corrected by the β-actin value of the same sample. The intraobserver variability was <5% and the results were further verified by a second blinded individual with an interobserver variability of <7% . The intensity of the actin band was considered in relation to that of the hnRNPA2/B1 in each specimen and arbitrarily assigned a plus (+) or minus (–) sign to indicate overexpression of hnRNPA2/B1. Border cases are indicated as +/–, whereas cases with higher levels of A2/B1 expression are indicated with ++ and +++.

Statistical analysis. The data collected were categorical and numerical. Categorical data were computed using a Microsoft Office Excel spreadsheet and compared with Chi-square analysis. Numerical data were analyzed as descriptive statistics and compared with *t*-test or Mann and Whitney test when normality

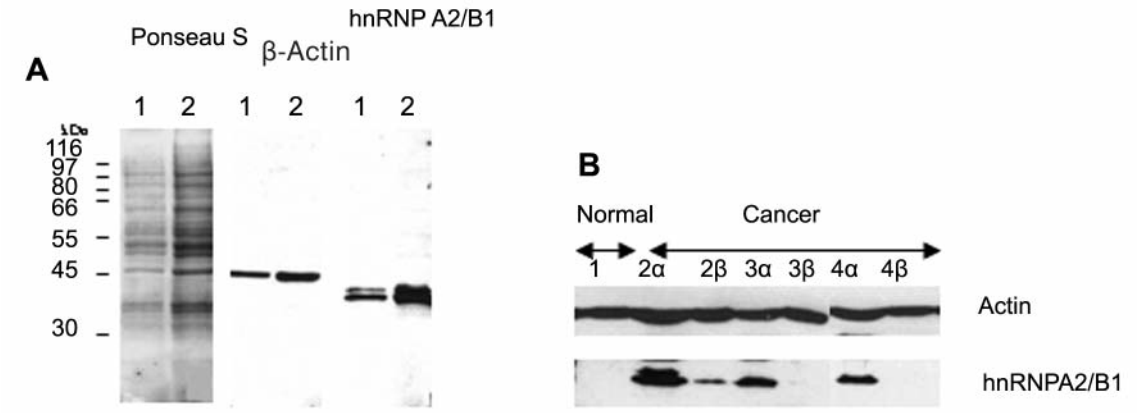

Figure 1. *Evaluation of the expression levels of hnRNPA2/B1 by immunoblotting. Panel A, Detection of hnRNPA2/B1 protein species in a total cell homogenate prepared from the lung cancer adenocarcinoma cell line A549 (1=4 μg and 2=10 μg total protein applied). Both actin and hnRNP A2/B1 protein species can be specifically immunodetected in low quantity of material, while the anti-hnRNPA2/B1 antibodies produced in our laboratory are able to recognize both hnRNP A2 and B1 spliced isoforms. Panel B, A representative picture corresponding to the expression levels of hnRNPA2/B1 relative to actin in surgically removed lung biopsies from the site of the lesion and adjacent normal appearing tissue of the same patient. Immunoblotting was performed on tissue homogenates obtained from lung cancer (lanes 2-4) and non-cancer (lane 1, normal) patients: α) site of cancer tissue, β) site of adjacent non-cancer tissue from the same patient. Lane 1 shows that normal adult lung tissue exhibited high levels of cellular actin whereas hnRNP a2/B1 was undetected. When homogenates from cancer and adjacent normal appearing tissue (sites α, β respectively) of the same biopsy were examined in pairs, hnRNP A2/B1 was overexpressed in the cancerous tissue. The level of observed overexpression varies among the biopsies tested, when normalized to cellular actin (lanes 2-4). In lane 2, where the highest expression level was observed, low levels of hnRNPA2/B1 were also detected in the adjacent normal appearing tissue.*

failed. The ANOVA method was used to detect any interactions between co-variants. The assumptions of the statistical analysis were met. The statistics were computed with SPSS statistical software.

Results

The validity of the sensitive immunochemical method of Western blotting and the sensitivity of the available antibodies was initially tested in a total cell homogenate prepared from the lung cancer adenocarcinoma cell line A549. As shown in Figure 1A, both actin and hnRNP A2/B1 protein species can be specifically immunodetected in low quantity of material. The immunoblotting protocol was firstly applied in total tissue homogenates obtained from surgically removed biopsies in order to verify whether it can accurately determine hnRNPA2/B1 antigen in this type of biological sample. Both actin and hnRNPA2/B1 protein levels were compared between surgically removed lung biopsies from the site of the lesion and adjacent normal appearing tissue of the same patient. Results are presented in Figure 1B. In all cases where homogenates from cancer and adjacent normal appearing tissue of the same biopsy were examined in pairs, overexpression of hnRNPA2/B1 was apparent in the cancerous tissue. In the pair with the highest expression level (no. 2), low levels of hnRNPA2/B1 were detected in the adjacent normal appearing tissue (lanes 2α, 2β).

Since we concluded that our immunoblotting approach could accurately determine overexpression of hnRNP A2/B1 antigen in surgically removed tissue from lung cancer patients, the blotting procedure presented above was applied in the analysis of bronchoscopic specimens. Thus, in cell or tissue homogenates made from either cell brushing or biopsies, respectively, the level of hnRNPA2/B1 expression was evaluated in relation to internal actin. In Figure 2, a representative immunoblotting is shown referring to a number of specimens of either cell brushings or biopsies.

Results concerning the clinicopathological characteristics of the study population are shown in Table I. Fifty-two out of 61 patients included in this study were diagnosed positive for lung cancer: 41 of these patients had non-small cell lung cancer (NSCLC) and 11 patients suffered from small cell lung cancer (SCLC), the remaining 9 patients were initially undiagnosed although they had undergone a full clinical, radiological and bronchoscopic control. More details about the study population are presented in Tables II, III and IV.

There was a statistically significant difference regarding the number of pack years (py) between lung cancer patients and controls ($p<0.001$). (Table V) There were no statistically significant age differences but male predominance was observed in lung cancer groups. hnRNPA2/B1 was overexpressed in lung cancer patients as compared to control subjects, regardless of the bronchoscopic specimen used (brushing or biopsy). In

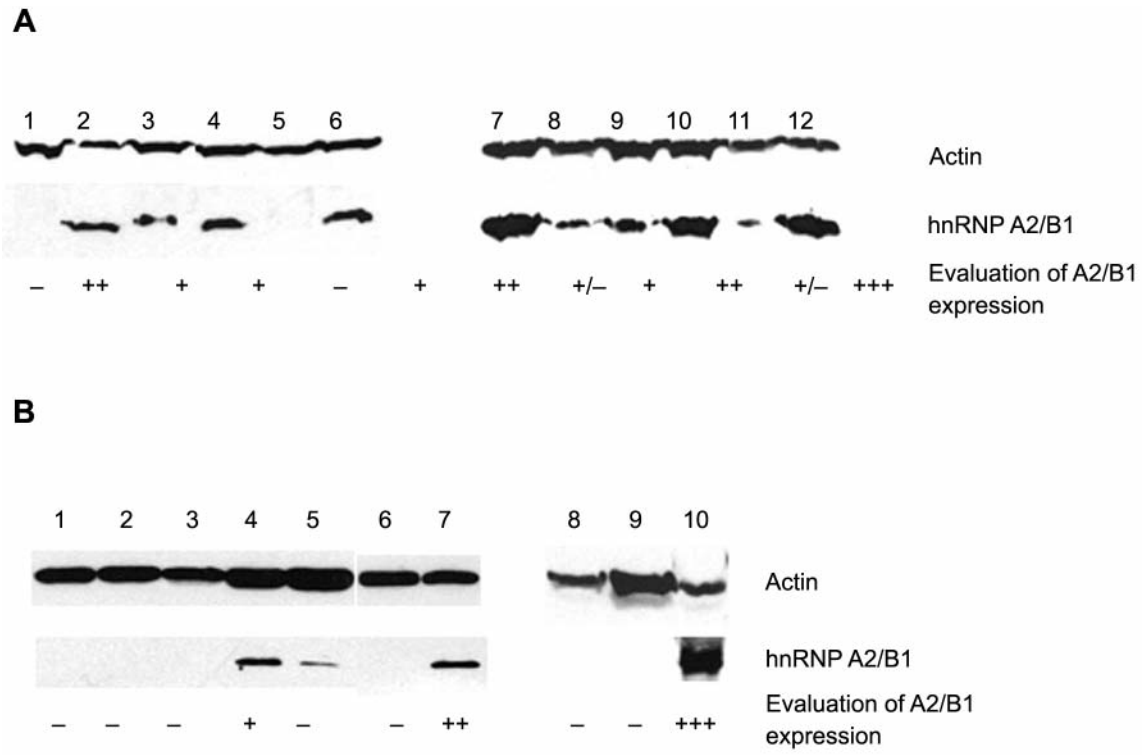

Figure 2. *Representative immunoblotting depicting the levels of hnRNPA2/B1 relative to actin for the evaluation of A2/B1. Panel A, specimens of cell brushings and Panel B, specimens of endobrochial biopsies.*

Table I. *Clinicopathothological characteristics of study population.*

Patients	No.	Diagnosis made by					Histology
		Biopsy	Brushing	Both	Other	FNA	
NSCLC	49	18	10	6	8	7	Squamous: 20 Non-differentiated: 18 Adenocarcinoma: 10 Neuroendocrine: 1
SCLC	12	4	2	3	2	1	SCLC: 12
Controls	30						No sign of malignancy

Other method: cytology of the pleural fluid, liver biopsy or Fine Needle Aspiration from a peripheral lesion of the lung (FNA).

Table VI we present percentages of hnRNPA2/B1 expression for every patient group. The frequency of histological subtypes in NSCLC patients is shown in Table VII. We were unable to detect any significant correlation between the expression of hnRNPA2/B1 and stage of the disease or histological type for the NSCLC patients. Using an intergroup ANOVA method, we found that age, gender and the number of cigarettes smoked (py) did not correlate with the expression of hnRNPA2/B1 in any patient group.

The sensitivity of hnRNPA2/B1 overexpression in patients suffering from NSCLC was 84.8% in brushing samples and 80.8% in biopsies. The sensitivity of hnRNPA2/B1 in patients with SCLC was 66.7% in brushing samples and 75% in biopsies. Overall, specificity of overexpression of this protein was 75.9% for brushing and 78.3% for biopsy. We concluded that the application of the immunoblotting approach was able to determine the overexpression of hnRNPA2/B1 antigen in brushing and biopsies from lung cancer patients, in all lung cancer subtypes, even in early stages (Table VIII). In SCLC

Table II. *Clinical data of the NSCLC study population, disease stage and tool used for diagnosis.*

Patient no.	Gender	Age (years)	Smoking (py)	Stage	Histological type	Diagnostic tool
001	M	81	80	IIB	non differentiated	endobronchial biopsy
002	F	66	0	IB	squamous	endobronchial biopsy
003	M	77	40	IV	non differentiated	endobronchial biopsy
005	M	63	100	IV	squamous	endobronchial biopsy
006	M	54	80	IIIB	non differentiated	cytology of pleural fluid
007	M	77	110	IV	non differentiated	washing
012	M	69	100	IA	squamous	FNA
013	M	49	90	IIIA	squamous	endobronchial biopsy
019	F	52	50	IB	squamous	FNA
020	M	55	80	IV	non differentiated	endobronchial biopsy
021	M	75	50	IIIB	adenocarcinoma	endobronchial biopsy, washing, brushing
025	M	70	10	IV	adenocarcinoma	biopsy of superclavicular lymphnode
026	M	72	40	IB	squamous	FNA
027	M	75	40	IB	squamous	washing
028	M	78	55	IV	non differentiated	washing, brushing
029	M	71	100	IIA	squamous	washing
030	M	72	150	IIIA	non differentiated	endobronchial biopsy
031	F	69	50	IV	adenocarcinoma	FNA
032	M	57	100	IB	non differentiated	washing, brushing
033	M	73	55	IA	non differentiated	endobronchial biopsy
034	M	43	100	IV	non differentiated	endobronchial biopsy
035	M	69	80	IIIB	non differentiated	FNA
042	M	67	140	IIIB	adenocarcinoma	cytology of pleural fluid
043	M	60	70	IB	squamous	brushing
044	F	59	60	IV	non differentiated	FNA
046	M	59	100	IV	non differentiated	brushing
050	M	70	20	IV	non differentiated	FNA
051	M	71	100	IV	adenocarcinoma	endobronchial biopsy
052	M	66	70	IV	squamous	endobronchial biopsy
054	M	50	130	IV	neuroendocrinal	liver biopsy
058	M	63	86	IIIB	adenocarcinoma	washing, brushing
059	M	52	35	IIIB	squamous	endobronchial biopsy, washing
060	M	57	80	IIIB	squamous	washing, brushing
065	M	68	40	IIB	adenocarcinoma	endobronchial biopsy, washing
069	M	78	100	IIIB	non differentiated	endobronchial biopsy
070	M	70	60	IV	non differentiated	endobronchial biopsy, washing, brushing
071	M	50	60	IB	squamous	endobronchial biopsy, washing
072	M	80	130	IIB	adenocarcinoma	endobronchial biopsy, washing, brushing
075	M	68	40	IV	squamous	endobronchial biopsy, washing, brushing
077	M	70	80	IV	adenocarcinoma	endobronchial biopsy
078	M	74	90	IV	non differentiated	washing, brushing
079	M	67	150	IIIA	non differentiated	washing, brushing
081	M	69	135	IIIA	squamous	endobronchial biopsy
082	M	70	40	IIB	squamous	endobronchial biopsy
084	F	75	0	IIIB	adenocarcinoma	endobronchial biopsy, washing, brushing
086	M	74	120	IIIB	squamous	endobronchial biopsy, washing, brushing
087	M	64	40	IIB	squamous	washing, brushing
090	M	26	1	IIIB	squamous	biopsy during surgery
091	M	67	150	IV	squamous	brushing

NSCLC: Non-small cell lung cancer, no.: number, M: male, F: female, py: pack years (age at presentation–age started smoking–years stopped)×(cigarettes/day), FNA: fine needle aspiration.

patients, we detected overexpression of hnRNPA2/B1 in 66% of brushing samples obtained from the contralateral lung (with no clinical, radiological or histological proof of disease, non-involved) and 66% of biopsies sites b, c (adjacent to the lesion and from the opposite lung respectively). As far as NSCLC patients are concerned, overexpression of this protein was detected in 79% of brushing samples obtained from the non-involved lung and in 81% of biopsies taken from sites b, c.

Table III. *Clinical data of the SCLC study population, disease stage and tool used for diagnosis*

Patient no.	Gender	Age (years)	Smoking (py)	Stage	Histological type	Diagnostic tool
004	M	61	80	extended	SCLC	brushing
011	F	81	3	extended	SCLC	endobronchial biopsy
014	M	68	50	extended	SCLC	endobronchial biopsy
018	M	67	15	extended	SCLC	endobronchial biopsy
024	M	73	100	extended	SCLC	endobronchial biopsy
040	M	54	35	extended	SCLC	endobronchial biopsy, washing, brushing
041	M	72	100	extended	SCLC	FNA
049	F	55	60	limited	SCLC	peripheral lymph node biopsy
055	M	77	50	limited	SCLC	FNA
061	M	75	40	limited	SCLC	washing
083	M	67	30	limited	SCLC	endobronchial biopsy, washing, brushing
088	M	64	60	limited	SCLC	endobronchial biopsy, washing, brushing

SCLC: Small cell lung cancer, no.: number, M: male, F: female, py: pack years (age at presentation–age started smoking–years stopped)×(cigarettes/day), FNA: fine needle aspiration.

In patients without lung cancer (controls), hnRNPA2/B1 was overexpressed in both brushings and biopsy in 5 patients, while in 2 patients the protein was overexpressed only in brushings. This subgroup of seven patients underwent clinical re-evaluation two and four years after they underwent bronchoscopy, all of them are healthy, but they remain under follow-up.

Eight of 61 patients were submitted to fine needle aspiration (FNA) in order to prove a diagnosis, as the usual cytological and histological examination of bronchoscopic samples were negative (Table I). However, in all of these patients, overexpression of hnRNPA2/B1 was detected in brushing and biopsies.

In 9 out of 61 patients, diagnosis was not made at the time of the first consultation, in spite of high clinical suspicion of malignancy. In this undiagnosed group, overexpression of hnRNPA2/B1 was detected in 7 out of 9 brushing samples and in 3 out of 5 biopsies, showing that this protein could serve as an early diagnostic tool when other methods failed to give a diagnosis. The follow-up of these patients 2 years after the sampling showed that 7 patients developed NSCLC and one of them SCLC. One patient was lost to follow-up.

Discussion

In spite of advances in early detection, the number of lung cancer patients diagnosed in advanced stages is increasing worldwide. In the present study, we investigated the hnRNPA2/B1 expression levels in brushing and biopsy specimens in patients with increased clinical probability for lung cancer, as well as in individuals without a malignant or any other disease (controls), in order to further evaluate its utility as a screening biomarker.

For the evaluation of the hnRNPA2/B1 expression, Western blot was used. This method is commonly used to identify a specific protein and to obtain qualitative and semi-quantitative data about it. It combines characteristics of immunology and biochemistry with satisfactory specificity and sensitivity. It is generally more sensitive and specific than the ELISA method. A major advantage is that the specific interaction between antibody and antigen can be directly visualized. It is a highly sensitive, specific technique which provides results within hours. There are several drawbacks, such as the fact that it is technically demanding, expensive and subject to interpretation concerning the presence and intensity of bands (22-23). The data are usually read visually (as in our study) or by densitometry and the strips are stable. Both visual inspection and densitometry are characterized by non linearity. This constitutes an additional disadvantage of Western blot because the result interpretation may be influenced by the absorption of protein and staining reagents by the blotting membrane (23-25). On the other hand, immunochemistry allows discrimination between tumor and normal cells and the use of routinely fixed embedded tissue samples. However, it is not considered a reliably standardized procedure. Western blot, therefore, is not an ideal method but a practical alternative to immunohistochemistry (22-23).

We must note that in our study, the percentage of non-differentiated large cell carcinomas was quite high (36.7%) without having used any specific patient selection criteria. According to literature, approximately 10-15% of lung cancer belongs to this histological subtype but this percentage has been reported higher in clinical practice. This could potentially be attributed to lung carcinoma histological heterogeneity (26).

It was shown that the level of hnRNPA2/B1 expression was significantly higher in brushing and biopsy samples

Table IV. *Clinical data of the control study population.*

Control no.	Gender	Age (years)	Smoking (py)
008	F	64	0
009	F	66	0
010	F	63	60
015	M	57	60
016	F	52	0
017	F	70	0
022	M	47	0
023	M	56	40
036	F	69	30
037	M	50	80
038	F	77	0
039	F	78	0
045	F	83	0
047	M	63	50
048	M	66	0
053	F	29	0
056	M	65	135
057	M	73	100
062	M	55	110
063	M	68	50
064	M	52	50
066	F	52	0
067	F	47	50
068	F	62	0
073	F	70	0
074	M	34	60
076	M	50	0
080	M	65	50
085	M	63	20
089	M	54	0

no.: number, M: male, F: female, py: pack years (age at presentation−age started smoking−years stopped)×(cigarettes/day).

Table V. *Descriptive statistics.*

	Age Mean (Range)	Gender M/F	Py Mean (SD)
Brushing			
SCLC	67.8 (54-81)	10/2	51.9 (30.4)
NSCLC	65.3 (26-81)	41/5	77.1 (40)
Control	60.5 (29-83)	16/13	30.8 (39)
Biopsy			
SCLC	66.7 (54-81)	6/2	52.8 (35.4)
NSCLC	67.7 (26-80)	24/2	74.4 (42.1)
Control	78.7 (34-78)	16/7	38.4 (40)

Table VI. *HnRNPA2/B1 expression levels in every patient group.*

	hnRNPA2/B1 expression (no. patients)
Brushing	
SCLC	8/12 (=66.7%)
NSCLC	39/46 (=84.8%)
Control	7/29 (=24.1%)
Biopsy	
SCLC	6/8 (=75%)
NSCLC	21/26 (=80.8%)
Control	5/23 (=21.7%)

Table VII. *Frequency of histological subtypes in NSCLC patients of the study population.*

NSCLC subtype	Frequency
Squamous cell	20/49 (=40.8%)
Non-differentiated large cell	18/49 (=36.7%)
Adenocarcinoma	10/49 (=20.4%)
Neuroendocrine	1/49 (=2.0%)

Table VIII. *hnRNPA2/B1 overexpression in early stages of lung cancer in the study population.*

	Stage I	Stage II
Brushing samples	7/9 (=77.8%)	5/6 (=83.3%)
Biopsies	4/5 (=80%)	2/2 (=100%)

when compared to specimens collected from adjacent normal appearing tissue from the same individual and from controls. Results concerning the detection of hnRNPA2/B1 in biopsy are comparable to published data (15), while no relevant data on brushings are available (9). Overexpression of hnRNPA2/B1 in bronchoscopic specimens seems to be a sensitive and specific marker for the early diagnosis of lung cancer (NSCLC, SCLC). No significant difference in the sensitivity and the specificity of the marker was observed between the two examined specimens, suggesting that brushing could also be used as the sole bronchoscopic method, since obtaining an endobronchial biopsy could sometimes be more difficult. It must be noted that hnRNPA2/B1 is a ubiquitously expressed protein, therefore, its importance as a tumor indicative marker lies in its overexpression.

There was no statistically significant association between hnRNPA2/B1 expression levels and age, histological type of tumour, grade of differentiation, stage of the disease. This agrees with previous reports (9, 27). It has been reported that correlation of smoking (py) with overexpression of hnRNPA2/B1 is stronger in SCLC than in NSCLC patients (28). In our study, this correlation was not statistically

significant for either SCLC or for NSCLC and this finding agrees with more recent published data (9). The most frequent histological subtype was squamous cell carcinoma. Overexpression of hnRNPA2/B1 was detected in all lung cancer subtypes.

It is important to note that hnRNPA2/B1 levels were elevated in stage I and II in brushing as well as in biopsy samples. This finding shows that hnRNPA2/B1 is overexpressed since the early stages of lung cancer, when therapy applied is more effective and life expectancy could be raised.

In controls, overexpression of hnRNPA2/B1 was detected in 24.1% of brushing specimens and 21.7% of biopsies. In previous reports, overexpression of this protein had been found in biopsy samples of normal lung tissue obtained from chronic smokers in 41% of cases (9). According to published data, molecular changes occur frequently in normal appearing lung tissue in central airways of chronic smokers and these can also be detected by hnRNPA2/B1 overexpression (9). This percentage of false-positive results limits the positive and negative predictive value of this marker. Nevertheless, it can be used for the detection of chronic smokers who are at greater risk of developing bronchial metaplasia and who are in need of close follow-up with a new bronchoscopic surveillance.

Along the same lines, in a considerable number of patients (66%), biopsies taken from an adjacent normal appearing area (b) as well as brushing and biopsy from the opposite lung (c) showed overexpression of hnRNPA2/B1, while histology and cytology revealed no abnormalities. The overexpression of this protein in morphologically and histologically normal (b) or (c) sites could be explained by the "field cancerization theory". According to this theory, initially proposed by Slaughter, the whole aero-digestive tract is exposed to carcinogens, such as tobacco and combustion products, and this exposure results in multiple foci of precancerous, highly proliferative cells (9, 15, 29). The overexpression of hnRNPA2/B1 found in morphologically normal sites of the lung might represent the widespread influence of inhaled carcinogens on the airway. The expression of hnRNPA2/B1 in tumours may not be homogeneous, with areas of both positive and negative expression (30-31). As far as the positive (c) site (contralateral non involved lung) is concerned, another possible explanation could be that the overexpression in the other lung appears earlier in time than in the tumour itself and it could be followed by cancerization, in an unknown time frame. Overexpression of hnRNPA2/B1 in biopsy samples of normal appearing tissue of the lung, adjacent to the lesion, was also detected in previous studies (9). Nonetheless, these findings point to the ability of detecting overexpression of the hnRNPA2/B1 at a precancerous, early stage, before the appearance of any cytological lesions and, therefore, strongly indicate that hnRNPA2/B1 could be a biomarker for early diagnosis.

In a number of lung cancer patients, hnRNPA2/B1 expression was absent in the site of the lesion or in adjacent lung tissue but was present in biopsies and brushings at a distance from the tumor. This limits the importance of hnRNPA2/B1 as a tumor-specific marker. The most likely explanation might be the presence of necrotic or hypoxic tissue at the site of the lesion or the nearby sites. Solid tumors frequently grow in low O_2 environments and Garayoa *et al*. have shown that sustained hypoxic treatment down-regulates the expression of hnRNPA2/B1 in a lung carcinoma cell line. This down-regulation was partially caused by hnRNPA2/B1 transcript destabilization (32).

A significant observation concerns the 8 patients who were submitted to FNA in order to be diagnosed, as the usual cytological and histological examination of bronchoscopic samples were negative. In all of these patients, overexpression of hnRNPA2/B1 was detected in brushing and biopsies, supporting the sensitivity of this diagnostic tool. As a diagnostic approach, bronchoscopy seems attractive since it is not very invasive, it does not require long hospitalization and it is not painful for the patient. Its contraindications and possible adverse events are infrequent and usually not serious as compared to FNA, open lung biopsy or other surgical procedures. In addition, it is inexpensive, allows samples to be obtained at an early stage and to simultaneously take histological and cytological samples in order to obtain a diagnosis, it is site - specific and its diagnostic yield is high.

The utility of hnRNPA2/B1 as an early detection marker in lung cancer is supported by the findings concerning hnRNP A2/B1 expression levels in 9 patients (out of 61) who underwent bronchoscopy but remained undiagnosed. The two-year follow-up of these patients showed that 7 of them had developed NSCLC and one of them SCLC. It is remarkable that overexpression of hnRNP A2/B1 was detected in 8 (out of 9) of these patients at the time of the first consultation (7 out of 9 brushing samples and 3 out of 5 biopsies), indicating that hnRNP A2/B1 protein was overexpressed even in the state of pre-malignant lesion.

hnRNPA2/B1 overexpression has previously been detected in several biological specimens (sputum, surgically removed tissue, endobronchial biopsy, bronchial lavage). The study in sputum concerned a particular population (patients at high risk of developing a second primary lung cancer and tin miners at high risk of primary lung cancer) (15). Although sputum is an easily obtained specimen and can show overexpression of hnRNPA2/B1, it has to be followed by bronchoscopy since the site of the lesion remains unknown. hnRNPA2/B1 expression levels has been studied only in endobronchial biopsy in chronic smokers and not in comparison to brushing samples,

obtained from the same patient (9). Recently, detection of hnRNP B1 mRNA in blood (plasma and serum) of lung cancer patients has been reported and thus, attempts have been made to use blood-based tests for early cancer identification, as a blood-based test would be more suitable for general application in clinical laboratories (16, 17 , 28). The fact that peripheral blood can be collected non-invasively and repeatedly in high-risk individuals renders this approach promising, especially since it has already been beneficial for the diagnosis of melanoma and breast cancer (33-34). The drawbacks of using the overexpression of hnRNPA2/B1 in blood samples as an early diagnostic tool is that it would be difficult to locate the organ affected by cancer (as overexpression is detected in various types of cancer) and prove the diagnosis. Moreover, RNA levels in bronchial lavage are much higher than those in serum (16). This finding supports that detection of this marker in bronchoscopic specimens is more helpful than its detection in serum. Furthermore, the usefulness of hnRNPA2/B1 detection in blood has yet to be proven. Perhaps in the future a combination of detection of hnRNP in bronchoscopic specimens and in blood could be beneficial (16, 17).

In conclusion, the immunoblotting results based on bronchoscopic specimens confirmed that hnRNPA2/B1 expression was markedly higher in lung cancer patients than in controls. It is important to note that overexpression was also detected in normal appearing tissue adjacent to the lesion and in the contralateral lung. Overall, these data suggest that hnRNPA2/B1 may be a sensitive and specific marker for the early detection of lung cancer in brushing and biopsy. Further investigation is needed in order to confirm its importance as a screening and diagnostic tool as well as to clarify the functions and clinical impacts of this protein and its significance in the prognosis of lung cancer.

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