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Altered patterns of expression of members of the heterogeneous nuclear ribonucleoprotein (hnRNP) family in lung cancer

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Summary hnRNP A2/B1 has been suggested as a useful early detection marker for lung carcinoma. hnRNP A2/B1 is a member of a large family of heterogeneous nuclear ribonucleoproteins (hnRNP proteins) involved in a variety of functions, including regulation of transcription, mRNA metabolism, and translation. In lung cancer, we have evaluated the expression and cellular localization of several members of the hnRNP family, hnRNP A1, A2, B1, C1, C2 and K. 16 cell lines (SCLC and NSCLC) and biopsies from 32 lung cancer patients were analyzed. Our results suggest that, besides hnRNP A2/B1, the expression of other members of the hnRNP family is altered both in SCLC and NSCLC. In the biopsies, negative or low expression of the hnRNP proteins analyzed was observed in normal epithelial cells whereas lung cancer cells showed highly intense nuclear or cytoplasmic immunolocalization. In all the lung cancer cell lines, the mRNA for all the hnRNP proteins was detected. In general, higher levels of hnRNP mRNAs were found in SCLC as compared with NSCLC. Our results also suggest that the expression and processing of each hnRNP protein in lung cancer is independently regulated and is not exclusively related to proliferation status. In SCLC cell lines, hnRNP A1 protein expression correlated with that of Bcl-x_L. In the lung cancer cell lines, hnRNP K protein localization varied with the cellular confluence.

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1. Introduction

The expression of a gene encoding a functional protein is initiated with the production of an immature pre-mRNA molecule. Virtually all mRNA molecules in eukaryotes are processed to some degree after they are synthesized. During their processing, the pre-mRNAs are subjected to modification in an operation controlled in a tissue- and developmental-specific manner. The normal functioning of this process is frequently altered in human diseases. It has been reported that 15% of human genetic diseases are caused by mutations that affect mRNA splicing [1]. In cancer, the expression of alternatively spliced mRNAs encoding altered forms of proteins has been related to tumorigenesis and tumor progression. BRCA1, Cyclin D1, mdm2, FHIT, TSG101, VEGF and CD44 are good examples of a rapidly growing list of cancer-related genes with aberrant splicing forms [2–7].

The mRNA processing, as well as other mRNA-related cell activities, are regulated by specific RNA-binding proteins such as the members of the hnRNP family [8]. About 20 major hnRNP proteins, from A1 to U, have been described and included in the family by their capacity to bind to pre-mRNA with no unique structural motif or function. Among the family, hnRNP A, D, E, I, and K have been reported to shuttle between the nucleus and the cytoplasm and some of them interact with each other forming hnRNP complexes [9]. The best characterized complex in mammalian cells is the 40S-ribonucleoprotein core particle associated to the pre-mRNA. hnRNP A, B and C represent the major proteins present in the core particle. They exist in a fixed molecular ratio forming apparently three different heterotetramers, $(A1)_3B2$, $(C1)_3C2$ and $(A2)_3B1$, although only two of them, $(C1)_3C2$ and $(A2)_3B1$, have been isolated and characterized [10,11]. Apart from this fixed stoichiometry, there is not a fixed set of hnRNP proteins that bind to every pre-mRNA. Rather, specific combinations of hnRNP proteins are thought to cluster on each type of pre-mRNA. This particular combination would depend on the mRNA sequence and the repertoire of hnRNP proteins present in the nucleus at the moment of transcription [8]. hnRNP proteins are involved in a variety of key cellular functions such as mRNA splicing [12], stabilization [13,14], nucleo-cytoplasmic transport [15–17], and transcriptional control [18]. Some of them can also control the transcription of specific genes [19,20]. Several evidences, summarized in the following paragraph, support the hypothesis that the hnRNP proteins may be relevant in human carcinogenesis.

Overexpression of hnRNP proteins has been reported in several types of cancers. hnRNP A1 is overexpressed in oligodendrogliomas [21] and chronic myelogenous leukemia [22]. hnRNP A1 expression is also increased in myeloid progenitor cells expressing the BRC/ABL oncoprotein. In these cells, an alteration of hnRNP A1 normal functioning results in a decrease of colony formation and tumorigenesis of these transformed cells, in part due to a downregulation of the antiapoptotic factor Bcl-x_L [22]. hnRNP A2 and hnRNP B1 are overexpressed in lung, breast, pancreatic and esophageal cancer [23–27]. In a retrospective study with sputum from high risk individuals, the use of a monoclonal antibody specific for hnRNP A2/B1 showed 88% specificity in the prediction of lung cancer 2 years in advance of any clinical evidence. Subsequent prospective studies have accurately predicted that 65% of individuals with a high immunoreactivity for this antibody in sputum would develop lung cancer in the first year of follow-up [26]. Using a polyclonal antibody specific for hnRNP B1, it has been suggested that hnRNP B1, and not hnRNP A2, is the protein overexpressed in early stages of lung carcinogenesis, although no biological explanation has been proposed for this selective overexpression [28,29]. The upregulation of hnRNP A2/B1 message in NSCLC has been associated with microsatellite instability, suggesting that lung tumor cells undergoing progression frequently overexpress hnRNP A2/B1 [30]. In addition, hnRNP subcellular localization seems to be an important factor associated with tumor progression, as cytoplasmic hnRNP A2/B1 immunoreactive cells have a higher frequency of microsatellite instability and loss of heterozygosity than do cells with nuclear immunoreactivity [31]. A study of the hnRNP A2/B1 expression during mammalian lung development revealed a regulated expression pattern of hnRNP A2/B1 during fetal development and downregulation in normal adult tissues [32]. This pattern of expression during development and the reexpression of the protein during tumor progression is consistent with hnRNP A2/B1 role as an oncofetal molecule. Although the biological reason for hnRNP A2/B1 overexpression in cancer is still not clear, it has been reported to be part of the molecular machinery that regulates telomere formation and/or stabilization [33], and has been also associated to the control of apoptosis [33,34]. These two functions may also be regulated by the other members of the 40S-ribonucleoprotein core particle, hnRNP A1 and hnRNP C1/C2 [33–35].

Other hnRNP protein potentially relevant in tumorigenesis is hnRNP K. In the nucleus, this protein can bind directly to the promoter region

of the human c-myc gene and functions as a transcription factor [19]. When localized to the cytoplasm, hnRNP K inhibits translation of specific mRNAs such as 15-lipoxygenase mRNA [36]. In breast cancer cells, hnRNP K significantly enhances cell proliferation and anchorage-independent growth through a growth factor dependent mechanism [37].

Several authors have reported an association of hnRNP expression with actively proliferating cells [38–40]. It is still not clear whether the overexpression of the hnRNP proteins in cancer is a tumor specific event or rather it is a mere consequence of the accelerated mRNA metabolism common to highly proliferative cancer cells. To clarify the relevance of the members of the hnRNP protein family in cancer, a systematic evaluation of the status of these proteins in normal versus tumor cells was required. It is also interesting to carry out detailed studies on the balance in the expression of splicing variants of individual hnRNP proteins, such as hnRNP A2/B1 and hnRNP C1/C2, in relation to carcinogenesis. Due to the diverse functions associated to each hnRNP protein (both in the nucleus and cytoplasm), other important aspect that may give light into the hnRNP functions implicated in lung cancer is the determination of the cellular localization of each particular hnRNP protein. To achieve these purposes, we decided to characterize in lung cancer the coordinated expression and distribution of several hnRNP proteins, rather than the isolated expression of a single one. We studied the expression of hnRNP A1, A2, B1, C1, C2 and K in normal lung epithelium, 16 lung cancer cell lines and 32 biopsies from lung cancer patients. Based on our results, we conclude that these hnRNP proteins are abnormally expressed in lung cancer as compared with normal respiratory epithelium. In the present paper we also discuss about the physiopathological consequences of this alteration.

2. Materials and methods

2.1. Cell lines

A range of lung cancer cell lines including eight NSCLC and eight SCLC cell lines (American Type Culture Collection, ATCC, Manassas, VA) were selected for the analysis. Cells were grown in the medium specified by ATCC (DMEM or RPMI 1640; Life Technologies, Inc., Carlsbad, CA) and supplemented with 10% fetal bovine serum and penicillin-streptomycin. Primary cultures of human bronchial epithelium cells (NHBE) grown in supplemented bronchial epithelial cell growth media (BGEM®, Clonetics, San Diego, CA) were also used. For all the cell lines, logarithmic phase growing cells were harvested at 70–80% confluence. For immunocytochemical techniques cells were processed differently depending on the optimal protocol implemented for each antibody. For hnRNP A1 and hnRNP C1/C2 cells were trypsinized (when adherent), pelleted, embedded in agarose, fixed in Bouin's fluid, embedded in paraffin and sectioned. Alternatively, for hnRNP A2/B1 and hnRNP K, adherent cells were grown directly on the surface of the microscope slides and then fixed in Saccomanno's fixative followed by acetone/methanol (1:1); non-adherent cells were pelleted, fixed as before and placed on microscope slides with the Cytospin® 3 cell Preparation System (Shandon, Pittsburg, PA).

2.2. Lung cancer biopsies

Lung carcinoma samples from stage I and II patients were obtained under an institution-approved human tissue procurement protocol. Tumors, adjacent areas to the tumor and distant (usually more than 5 cm) normal uninvolved tissue were obtained and analyzed. Samples were consistently immersed in buffered formalin within 20 min from surgical resection. All the samples were removed from the fixative solution after 24 h fixation. Tissues were then paraffin embedded and sectioned. A total of 30 NSCLC cases were studied, including 15 squamous cell carcinomas, ten adenocarcinomas, four bronchioloalveolar tumors, and one carcinoid. Samples from two patients that were diagnosed with SCLC after pathological analysis of the surgical pieces were also included in this series. Patients ranged from 42 to 87 years of age, all of them were current or former smokers except for one. Sex distribution was five females and 27 males.

2.3. Antibodies

The mouse monoclonal antibody (MoAb) 703D4 (IgG2bK) specific for hnRNP A2/B1 was a kind gift from Dr J.L. Mulshine (National Cancer Institute, Bethesda, MD). The antibodies against hnRNP A1 (4B10; IgG2a) and hnRNP C1/C2 (4F4; IgG1) both MoAb were a kind gift from Professor G. Dreyfuss (Howard Hughes Medical Institute, Philadelphia, PA). The affinity purified-polyclonal antibody against hnRNP K was developed by Dr F. Lecanda (University of Navarra, Pamplona, Spain). The antibodies against two splicing forms of one single gene (A2/B1 and C1/C2) were directed against the common sequences of both proteins. The rabbit

polyclonal antibody against Bcl-$x_{S/L}$ was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The specificity of each antibody was confirmed by Western blot analysis. For immunocytochemistry the working dilutions for the hnRNP antibodies were: 703D4, 1/100; 4B10, 1/6000; 4F4, 1/6000; and for the antibody against hnRNP K, 1/50 for the cell lines and 1/100 for the lung biopsies. For Western blot analysis, the dilutions of the antibodies were: Bcl-$x_{S/L}$, 1/1000; 4B10, 1/2000, and hnRNP K, 1/1000.

2.4. Immunocytochemistry

Sections (3 μm) were deparaffinized and incubated overnight at 4 °C with the corresponding antibody. Only the biopsies incubated with the antibody against hnRNP K needed previous treatment with citrate buffer and microwave oven. The next steps of the protocol were optimized for each one of the antibodies used as follows. For the 4B10 antibody, the ENVISION® (DAKO, Carpinteria, CA) system was applied for 30 min; for 703D4, we used universal secondary antibody containing rabbit anti-mouse IgG (DAKO) for 30 min followed by incubation with ABC complexes (DAKO) for 30 min for the cell lines, and the ENVISION® (DAKO) system (30 min) was applied for the lung biopsies. In the case of 4F4 antibody, universal secondary antibody containing rabbit anti-mouse IgG (DAKO) (30 min) and ABC complex (30 min) were used. Finally, for the antibody against hnRNP K, we also used ENVISION® (DAKO) (30 min). The immunostaining was then developed with diaminobenzidine and counterstained with hematoxylin. Negative controls by omission of primary antibodies were done for all the cases.

2.5. Western blot analysis

Total protein extracts from cell lines were electrophoretically fractionated on 4–12% Bis-Tris gels (Novex, San Diego, CA), transferred to a 0.2 μm nitrocellulose membrane, and blocked with 5% milk in PBS. Afterwards the membrane was incubated with the primary and secondary antibodies and developed using the Lumi-Light^{PLUS} Western Blotting Kit (Roche, Indianapolis, IN) according to the manufacturer's instructions.

2.6. Real-time quantitative reverse transcription-PCR

Expression of the hnRNP A1, A2, B1, C1, C2 and K genes were characterized by real-time quantitative reverse transcription-PCR with the ABI PRISM™ 7700 Sequence Detector and the software Sequence Detector version 1.6.3. (Perkin–Elmer/Applied Biosystems, Foster City, CA). RNA was isolated from all the cell lines with the GIT/Cesium Chloride method. The reactions were performed with 2 μg total RNA with minor differences from ABI 7700 manufacturer's instructions. Relative levels of the hnRNP mRNA proteins studied expression were determined by the Ct method. Every assay was performed in triplicate and two different samples obtained in separate experiments were analyzed for each cell line. Primers for hnRNP mRNA amplification were: hnRNP A1 forward 5′-TCGTCAGCTTGCTCCTTTCTG-3′, reverse 5′-ATGACGGCAGGGTGAAGAGA-3′; hnRNP A2 forward 5′-TCTCTCTCATCTCGCTCGGC-3′, reverse 5′-CTTACGGAACTGTTCCTTTTCTCTCT-3′; hnRNP B1 forward 5′-TCTCTCTCATCTCGCTCGGC-3′, reverse 5′-CGGAACTGTTCCTTTTCTCTCTTT-3′; hnRNP C1 forward 5′-AAAAGCAGGTGTGAAACGATCTG-3′, reverse 5′-GGAGGTACACGTGCTGGGTAA-3′; hnRNP C2 forward 5′-GCGGAGATGTACGGGTCAGTA-3′, reverse 5′-GGAGGTACACGTGCTGGGTAA-3′; hnRNP K forward 5′-CAGCATTGCAGACGCCATTAT-3′, reverse 5′-GTGGGACACAGGCAAGACGT-3′. The TaqMan probes for the amplified sequences detection were: hnRNP A1 5′-(6-Fam)CCGCCGAAGAAGCATCGTTAAAGT-(Tamra)(phosphate)-3′, hnRNP A2/B1 5′-(6-Fam)AAATCGGGCTGAAGCGACTGAGTCC(Tamra)(phosphate)-3′, hnRNP C1 5′-(6-Fam)CGGAGATGTACGGCTCCTCTTTTGA-(Tamra)(phosphate)-3′, hnRNP C2 5′-(6-Fam)CAGAACACCCTTCTCCGTCCCCT-(Tamra)(phosphate)-3′ and hnRNP K 5′-(6-Fam)-CTGTTTCTCTGCTGCACCGACCTC-(Tamra)(phosphate)-3′. All experiments included a GAPDH internal standard. The primers used for GAPDH amplification were 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGATTTC-3′. The probe for GAPDH detection was 5′-(Joe)CAAGCTTCCCGTTCTCAGCC-(Tamra)(phosphate)-3′. Quantitative mRNA levels for each hnRNP gene were expressed as relative (percentage) comparison to a housekeeping gene (GAPDH) mRNA.

2.7. Statistical analysis

The values obtained from real-time RT-PCR were analyzed by *U* Mann–Whitney test to compare data between SCLC and NSCLC cell lines. Correlations in data obtained from the biopsies were analyzed by Kendall's correlation test. A P value less than 0.05 was considered to be significant.

3. Results

In this study we have analyzed the expression of different hnRNP proteins in a variety of lung cancer cell lines both SCLC and NSCLC as well as in normal, hyperplastic and malignant lung tissues. We quantified the gene expression of each hnRNP protein in eight SCLC and eight NSCLC cell lines by real-time PCR. The expression levels of each hnRNP protein were assessed for the same cell lines by immunocytochemistry. For each cell line, we have obtained a comparative value denoted ΣhnRNP, consisting in the addition of the mRNA value from each hnRNP protein analyzed. Cell line to cell line, ΣhnRNP varied ranging from 35.6 (H774) to 129.9 (H510) in SCLC and from 19.3 (A549) to 33.8 (H676) in the case of NSCLC. Overall, the average expression of the hnRNP mRNAs in SCLC was significantly higher ($P < 0.001$) than that in NSCLC (average levels were 75.0 ± 37.1 and 26.2 ± 4.9, respectively). Finally, we characterized by immunocytochemistry the expression of these proteins in a series of biopsies from patients diagnosed for lung cancer, mainly non-small cell lung cancer at early stages (stages I or II). Fig. 1 and Table 1 summarize the expression of hnRNP proteins in the biopsy samples.

3.1. hnRNP A1

hnRNP A1 was detected in all of the cell lines (SCLC and NSCLC) analyzed. Average mRNA expression in SCLC cell lines was 4.3 fold higher than that in NSCLC cell lines (average mRNA percentage level 13.7 ± 6.4 and 3.2 ± 1.5 respectively, $P = 0.006$), although not all SCLC cell lines (i.e. N417 and H774) presented higher levels of expression than the NSCLC cell lines. H69, H82 and H510 showed the highest levels of hnRNP A1 message (Fig. 2). In every cell line, the immunocytochemical analysis of sectioned cultured cells revealed a strong hnRNP A1-like staining localized in the nucleus. As a representative example, Fig. 1e shows hnRNP A1 staining of H1264 cell line. Western blot analysis in protein extracts from SCLC cells lines also revealed a high expression of hnRNP A1 (Fig. 3). Interestingly, in most cell lines studied a parallel expression of hnRNP A1 and Bcl-x_L was observed (Fig. 3), with the clear exception of H446. No Bcl-x_S expression was detected in any of the SCLC cell lines analyzed. In biopsies, hnRNP A1 was consistently detected both in normal and tumor areas (Table 1 and Fig. 1a–d). No immunoreactivity for hnRNP A1 was detected in the alveolar epithelial cells (Fig. 1a), except in the areas of thickened (fibrotic) septa where we observed a moderate epithelial nuclear staining. Normal bronchiolar epithelium showed hnRNP A1 moderate nuclear localization in a large proportion of the cells (Fig. 1b). Interestingly, an increase in hnRNP A1 expression was consistently observed in every area of hyperplastic type II pneumocytes, which showed strong nuclear staining for the protein (Fig. 1c). In bronchiolar hyperplasias, the intensity of hnRNP A1 nuclear staining was higher than that found in normal bronchiolar cells. All NSCLC biopsies showed a very marked nuclear staining in almost 100% of the tumor cells (Fig. 1d). In general, the normal tissue adjacent to the tumor expressed higher levels of hnRNP A1 than the distal unaffected normal tissue. In the case of the two SCLC samples, an intense nuclear staining was observed in all the tumor cells.

3.2. hnRNP A2/B1

Our primer design for real-time RT-PCR allowed us to distinguish between the two transcripts (A2/B1) arising from the same gene. All the lung cancer cell lines analyzed expressed both hnRNP A2 and hnRNP B1 mRNA. Expression for both transcripts in SCLC cell lines was almost invariably higher than in NSCLC cell lines (hnRNP A2: $P = 0.001$; hnRNP B1: $P = 0.009$), except for hnRNP B1 in H1385 that was similar to NSCLC levels. Overall, the average expression of hnRNP A2 mRNA was 7.2 times higher than that of hnRNP B1 in the case of SCLC (average mRNA percentage level 25.3 ± 7.9 and 3.5 ± 1.7, respectively) and 6.3 fold in the case of NSCLC (average mRNA percentage level 7.5 ± 2.4 and 1.2 ± 0.8, respectively) (Fig. 4). Cell line to cell line hnRNP A2 to hnRNP B1 ratio varied ranging from 12.1 (N417) to 3.8 (H69) in SCLC and from 11.8 (H1264) to 3.1 (H1385) in NSCLC, without significant statistical differences between SCLC and NSCLC. In all the cell lines studied hnRNP A2/B1-like immunoreactivity was primary located to the cytoplasm (Fig. 1j). By immunocytochemistry, normal lung alveoli and bronchioli were negative for hnRNP A2/B1 protein (Table 1, Fig. 1f and g). Half of the cases showed positive cytoplasmic staining in the hyperplastic epithelial tissue. While in the alveolar hyperplastic type II pneumocytes a strong staining was localized in a granular pattern, the staining of hyperplastic bronchioli was moderate, less common and also granular. About 60% of the NSCLC biopsies showed a moderate patched cytoplasmic staining of moderate intensity in tumors (Fig. 1i). hnRNP A2/B1 expression was more frequent in adenocarcinomas than in squamous cell carcinomas (79 and 44%, respectively, $P = 0.036$). Besides, our data indicates that hnRNP

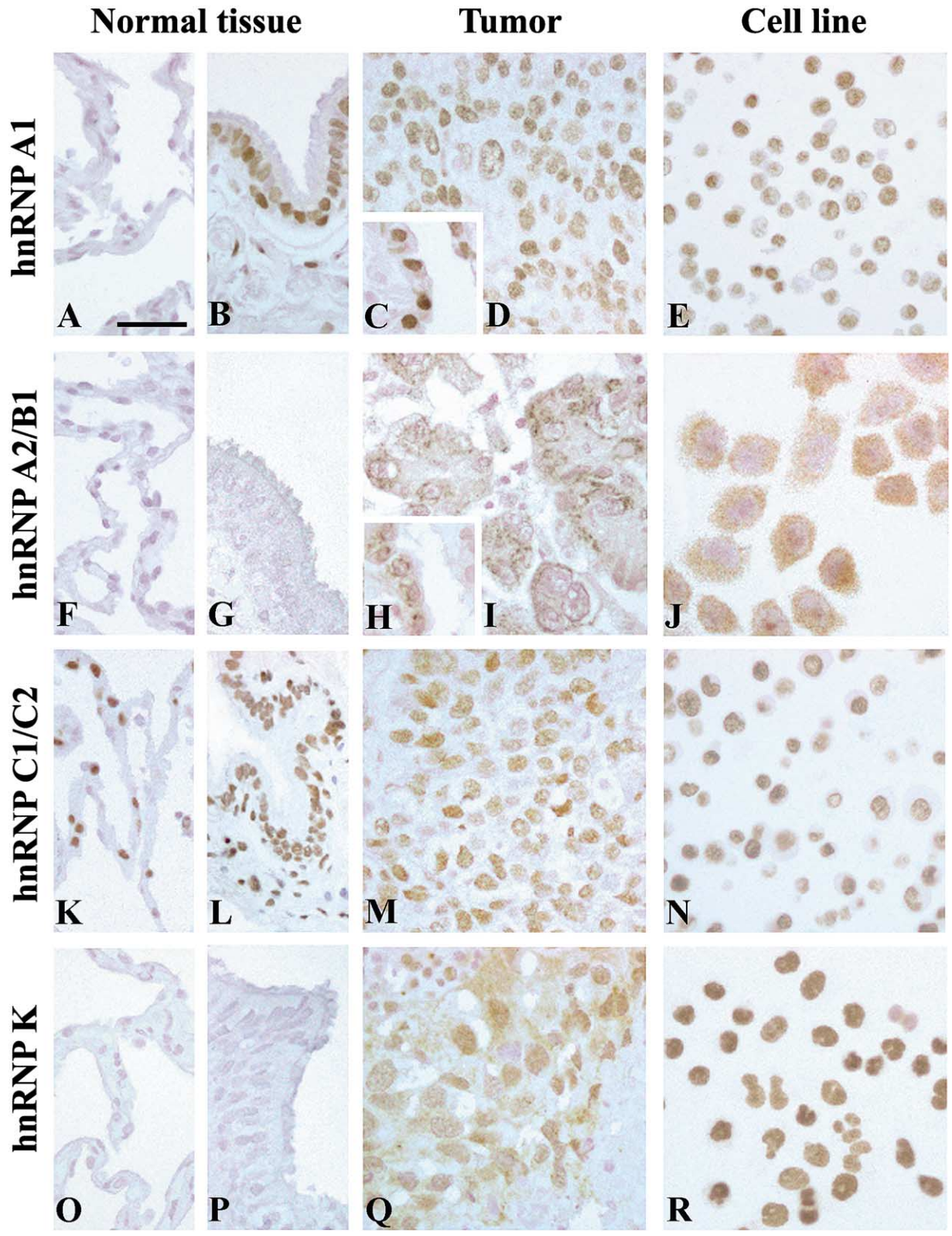

Fig. 1 Expression of hnRNP proteins is increased in human lung cancer when compared with normal tissues as studied by immunocytochemistry. The figures include representative examples of the expression of the five hnRNP proteins in tissues and cell lines. (a–e) hnRNP A1 protein expression. (f–j) hnRNP A2/B1 protein expression. (k–n) hnRNP C1/C2 protein expression. (o–r) hnRNP K protein expression. (a, f, k, and o) Normal alveolar cells. (b, g, l, and p) Normal bronchiolar cells. (c and h) Type II pneumocyte hyperplasia. (d, i, m, and q) NSCLC tumor cells. (e, j, n, and r) H1264 NSCLC cell line. Original magnification of all the pictures: $\times$ 500, scale bar = 50 μm.

Table 1 Expression and localization of hnRNP proteins in 30 normal human lung and NSCLC biopsies by immunocytochemistry

	hnRNP A1	hnRNP A2/B1	hnRNP C1/C2	hnRNP K
Normal alveoli	−[a]	−	N[b] + + (50%)[c]	−
Normal bronchioli	N + + + (75%)	−	N + + + (50%)	−
Type II pneumocyte hyperplasia	N + + + (75%)	C + + + (75%)	N + + + (75%)	N + (25%)
Bronchiolar hyperplasia	N + + + + (75%)	C + + (50%)	N + + + (75%)	N + + (50%)
Tumor	N + + + + (100%)	C + + (75%)	N + + + + (100%)	N + + + (75%)[d], C + + + (50%)

[a] Intensity of staining: − negative; + low; + + moderate; + + + high; + + + + very high.
[b] N, nuclear staining; C, cytoplasmic staining.
[c] In parenthesis, percentage of stained cells in the positive tissues.
[d] Two entries for hnRNP K in tumors are included due to the double localization of the protein, both in the nucleus and the cytoplasm.

A2/B1 is preferentially expressed by well or moderately differentiated tumors, concluding that there exists a correlation ($P = 0.011$) between histological grade and protein expression: 100% of well differentiated tumors ($n = 4$) expressed hnRNP A2/B1, 67% of moderately differentiated tumors ($n = 15$) expressed the protein and only 37% of the poorly differentiated tumors ($n = 8$) presented hnRNP A2/B1 expression. One of the two SCLC cases included in the study was negative for hnRNP A2/B1 while the other showed moderate nuclear and cytoplasmic staining.

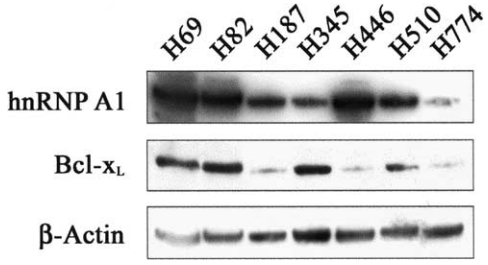

Fig. 3 Comparison of hnRNP A1 and Bcl-x_L expression in various SCLC cell lines. β-Actin expression was used to assess equal loading.

3.3. hnRNP C1/C2

hnRNP C1 and hnRNP C2 were expressed by all the lung cancer cell lines analyzed. Our design for real-time PCR allowed us to distinguish between the two transcripts, hnRNP C1 and C2. The mRNA analysis showed a consistently and remarkably higher expression of hnRNP C1 when compared with hnRNP C2 in every cell line from both SCLC and NSCLC (Fig. 5). In SCLC the hnRNP C1/C2 mRNA ratio ranged from 170.9 (H69) to 7.9 (H82), in NSCLC C1/C2 ratio differed from 162.1 (H676) to 4.0 (HTB55 and H1264). Levels for hnRNP C1 and C2 mRNA were the highest in H510. hnRNP C1 mRNA expression was higher ($P = 0.004$) in SCLC cell lines (average mRNA level 9.7 ± 5.5) than in NSCLC cell lines (average level 3.3 ± 1.4). Such differential expression was not found for hnRNP C2 mRNA (average mRNA level 0.5 ± 0.4 and 0.4 ± 0.3, respectively, for SCLC and NSCLC cell lines). In all the cell lines studied a strong nuclear hnRNP C1/C2-like immunostaining was observed (Fig. 1n). In the biopsies studied, both normal and tumor lung epithelial cells were immunoreactive for hnRNP C1/C2 (Table 1 and Fig. 1k–m). Normal alveolar and bronchiolar tissue showed moderately intense nuclear reactivity in 70% of the cases. The epithelial hyperplastic lesions were more intensely stained than normal tissue and less than the tumors. 100% of the non-small lung tumors studied displayed a very strong nuclear staining that extended to the total area of the tumor. This

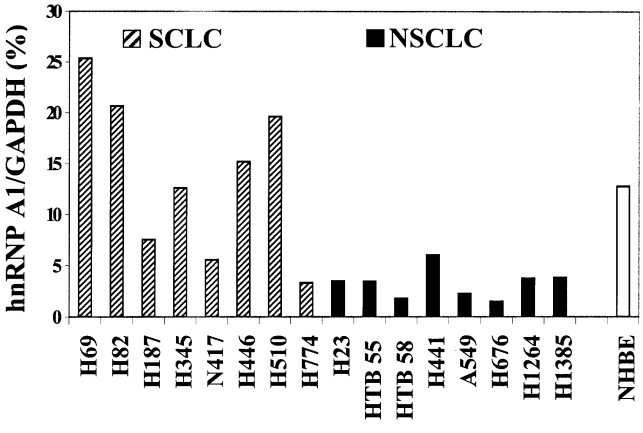

Fig. 2 Expression of hnRNP A1 mRNA in lung cancer cell lines by real time RT-PCR. The *y*-axis represents the relation between hnRNP A1 and GAPDH mRNAs in percentage.

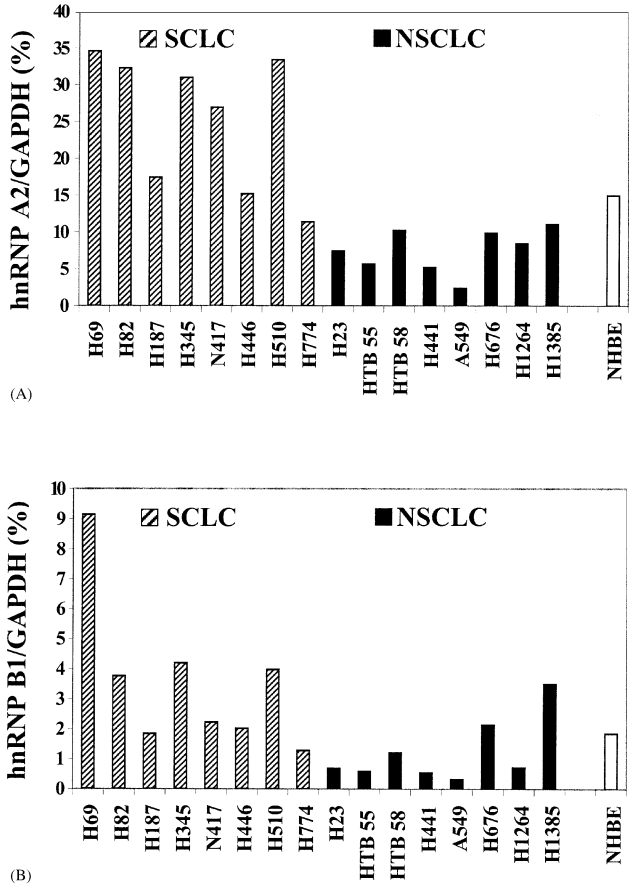

Fig. 4 Expression of hnRNP A2 (A) and hnRNP B1 (B) mRNAs in lung cancer cell lines by real time RT-PCR. The *y*-axis represents the relation between hnRNP A2 or B1 and GAPDH mRNAs in percentage. Note that the scale is different between the two graphs.

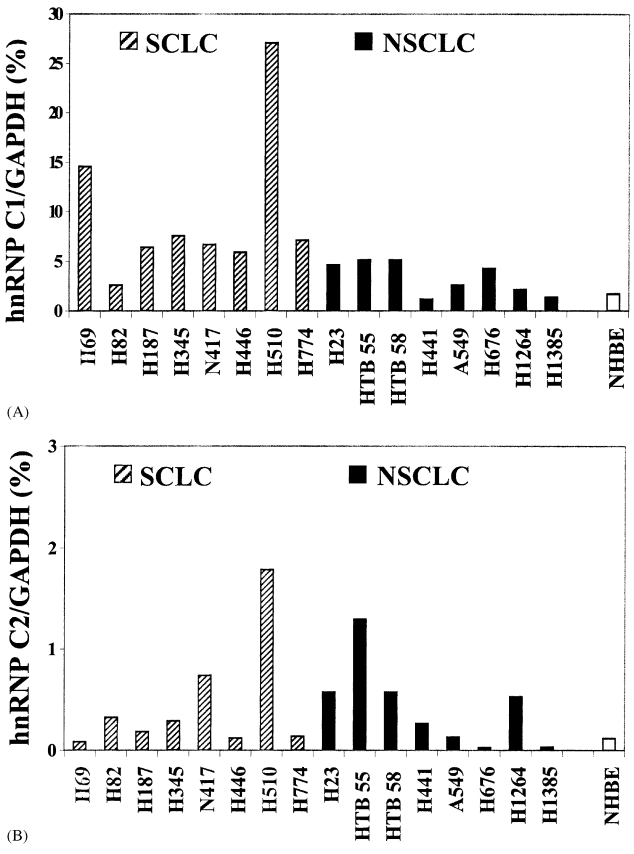

Fig. 5 Expression of hnRNP C1 (A) and hnRNP C2 (B) mRNAs in lung cancer cell lines by real time RT-PCR. The *y*-axis represents the relation between hnRNP C1 or C2 and GAPDH mRNAs in percentage. Note that the scale is different between the two graphs.

intense nuclear staining was also observed in the two SCLC biopsies.

3.4. hnRNP K

hnRNP K was expressed in every cell line analyzed. mRNA expression in SCLC cell lines (average level 22.3 ± 14.9) seemed to be higher than that in NSCLC cell lines (10.5 ± 2.6) (Fig. 6), although the difference did not reach statistical significance. H69, N417 and H510 were the highest hnRNP K expressing cells among the SCLC lines. In log phase growth, all cell lines showed high nuclear hnRNP K immunostaining (Fig. 1r). This pattern shifted in cells analyzed from confluent cultures, where the expression was also found in the cytoplasm and higher than in log phase growing cells (Fig. 7). In all clinical specimens studied, normal lung alveoli and bronchioli were negative (Table 1, Fig. 1o, and p). In bronchiolar hyperplasia there was a moderate increase in hnRNP K expression that was almost not detectable in type II pneumocyte hyperplasia. Remarkable overexpression of hnRNP K was observed in 60% of the tumors studied with a predominant expression in adenocarcinomas versus

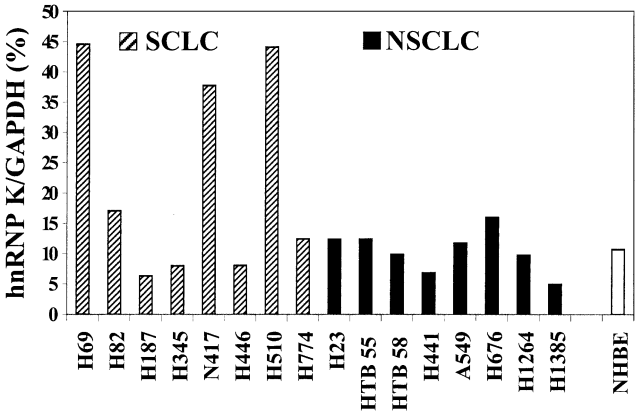

Fig. 6 Expression of hnRNP K mRNA in lung cancer cell lines by real time RT-PCR. The *y*-axis represents the relation between hnRNP K and GAPDH mRNA in percentage.

squamous cell carcinomas (79 and 33%, respectively, $P = 0.006$). In these cases the localization of the protein was mostly nuclear, but half of the positive cases showed also cytoplasmic immunostaining (Fig. 1q). In the two cases of SCLC studied only nuclear localization of the protein was found.

4. Discussion

In this work we have characterized the expression and distribution in lung cancer of several hnRNP proteins involved in mRNA metabolism and with potential implications in carcinogenesis. We conclude that the expression of these proteins is modified in lung cancer as compared with normal lung epithelium and, therefore, suggest that the machinery for mRNA metabolism is altered in lung carcinogenesis. Besides, this abnormal expression is not a mere consequence of activated proliferation and can be already observed in preneoplastic lesions. This interesting fact suggests a potential use of these molecules, and possibly other mRNA processing proteins, in early detection of lung cancer.

To date there is not a single fully validated biomarker for early detection of lung cancer. During the past years a lung cancer-associated monoclonal antibody that recognizes hnRNP A2/B1 [40] has been proposed as a marker for this purpose. In an attempt to improve our knowledge on the implication of hnRNP A2/B1 and other important hnRNP proteins in lung carcinogenesis, we have characterized the combined expression pattern and location of hnRNP A1, A2/B1, C1/C2 and K in cancer cell lines as well as normal lung and early stages of lung cancer. Taken together, our results show marked differences in the expression of the hnRNP proteins between normal lung epithelium and lung tumors. In all cases, an overall higher expression of the hnRNP proteins was observed in tumors when compared with normal lung bronchiolar and alveolar epithelial cells. Higher hnRNP mRNA levels were found in SCLC cell lines as compared with most NSCLC cell lines. As an indication of the activation of the mRNA processing machinery in each cell line, we have

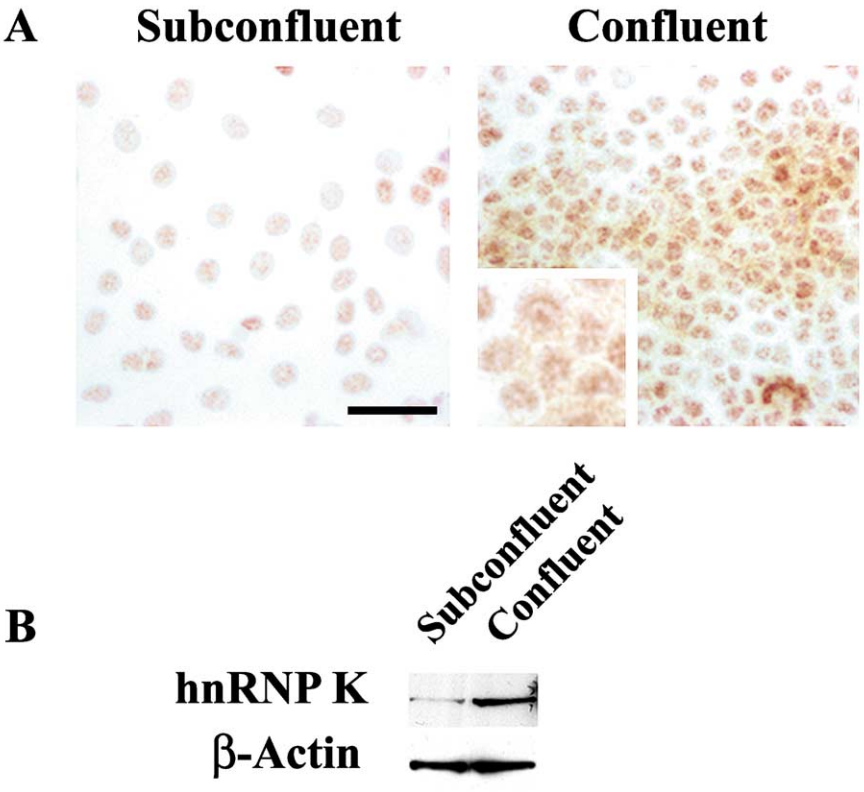

Fig. 7 Nuclear expression of hnRNP K in H460 cells increases from non-confluent to confluent cultures. In confluent cells hnRNP K protein is also found in the cytoplasm. Data shown are a representative result of three independent experiments. (A) Immunocytochemical analysis. Magnification: × 500 (inset × 1250); scale bar = 50 μm. (B) Western blot analysis. β-Actin expression was used to assess equal loading.

obtained a comparative value denoted ΣhnRNP, consisting in the addition of the mRNA value from each hnRNP protein analyzed. The fact that SCLC cell lines consistently have higher ΣhnRNP suggests that these types of tumors are more actively involved in mRNA processing than NSCLC probably due in part to a higher proliferative capacity of SCLC cells when compared with NSCLC cells. The results obtained with the normal bronchial epithelial cells NHBE seem to confirm the already suggested relationship between proliferation and increase in the expression of members of the hnRNP family [38–40]. These proliferating cells show moderately high levels of hnRNP mRNAs. In parallel, we have also observed in the biopsies an increase of the hnRNP protein immunolocalization in hyperplastic epithelial areas. However, our results also indicate that the increase in the expression of hnRNP proteins due to proliferation does not explain by itself the altered pattern of expression of hnRNP proteins in lung cancer cell lines and tissues. This can be exemplified by the fact that in each cell line there is no correlation between the levels of all hnRNP proteins. Some cell lines present high levels of a particular hnRNP protein but relatively low levels (compared with other cell lines) of other hnRNP proteins. For example, H82 cell line shows high levels of hnRNP A2, relatively low levels of hnRNP C1, and moderate levels of hnRNP C2 and hnRNP K. If only cell proliferation had accounted for hnRNP protein overexpression we would have expected a correlative increase in the levels of all hnRNP proteins from low to high proliferative index, which is not the case. Moreover, within NSCLC cases, we have found a significant correlation between the expression of some hnRNP proteins and the differentiation grade that would rather suggest an inverse correlation of these hnRNP proteins and proliferation. In summary, our results show that, although part of the increase on hnRNP expression may be due to the high metabolic rate characteristic of the cycling cells, this activation does not explain by itself all the alterations in the expression of these mRNA processing molecules in lung cancer. All of that is in agreement with previous works in which hnRNP A1, hnRNP A2/B1 and hnRNP C1/C2 were less abundant in normal tissues compared with transformed cell lines but only a partial relation between rate of cell proliferation and expression of hnRNP proteins was found [39].

The relative expression level of the hnRNP proteins involved in the 40S-ribonucleoprotein particle varies from cell line to cell line. This finding suggests that in different types of tumors there are alterations from the ''canonical'' proportion of these proteins: heterotetramers $(C1)_3C2$ and $(A2)_3B1$. This stoichiometry seems important for the function of the 40S complex and a modification in the relative levels of each hnRNP protein, such as what we observe in malignant lung cells, may alter the processing of the different pre-mRNAs controlled by these complexes [41]. Interestingly, the pattern of expression of the five hnRNP proteins is also different when both SCLC and NSCLC cell lines are compared. These variations in the pattern of hnRNP expression may be relevant to the processing of specific mRNAs in each cell line. More importantly, the stoichiometry for the hnRNP proteins coming from the same gene (A2/B1, C1/C2) varies from cell line to cell line, indicating that there may also exist a regulation in the splicing of the hnRNP gene itself that could be thus relevant to its role in the mRNA processing of other genes. This complex scenario may also apply to hnRNP A1, for which it has been shown that mRNA splicing can be regulated by its own protein [42]. Therefore, to understand the role of hnRNP proteins in cancer, we need to determine, not only the expression of every gene but also the relative proportion of its diverse alternative splicing forms.

Our results support previous reports showing a high cytoplasmic expression of hnRNP A2/B1 in primary NSCLC as well as in hyperplastic lesions [32,43]. Our data also extend the potential relevance of hnRNP A2/B1 to SCLC early detection, as the mRNA levels of this protein seem to be particularly high in SCLC. A further finding is that hnRNP A2/B1 expression is related to the histological type of the tumor and the grade of differentiation. Besides, its expression in the tumors is not homogeneous, with areas of both positive and negative expression. The biological reason for that is unknown although it would be of interest to determine the correlation between the expression of hnRNP A2/B1 and other sources of tumor heterogeneity such as the degree of neoangiogenesis and the areas of tumor hypoxia. Recent data indicate that hnRNP A2/B1 expression is downregulated in lung cancer cells under hypoxia [44]. Our data also suggest that hnRNP A2/B1 is not the only RNA binding protein associated with lung carcinogenesis. Several of the components of the RNA processing machinery seem to be overexpressed in lung cancer and this alteration may be involved in the process of lung carcinogenesis. In this sense, we have demonstrated a progressive increase in nuclear hnRNP A1 expression from normal lung epithelium, to hyperplastic lesions (both bronchiolar and alveolar), and to tumors. A similar trend was found for nuclear immunoreactive hnRNP C1/C2 and hnRNP K.

hnRNP K is the only hnRNP protein analyzed in the present study that is not part of the 40S-ribonucleoprotein complex. We have shown a noticeable nuclear overexpression of hnRNP K in tumors when compared with normal lung, with moderate intensity levels in some hyperplastic lesions. In the nucleus, hnRNP K binds to the promoter region of the human c-myc gene and functions as a transcription factor [19]. Our results show that the four SCLC cell lines with highest hnRNP K mRNA levels are those for which amplification of at least one of the myc genes has been reported, whereas no amplification of any myc gene has been found in three of the four SCLC cell lines that have lower hnRNP K expression. Further studies may clarify whether regulation of the expression of hnRNP K gene is controlled by the degree of amplification of myc gene. A further finding is that hnRNP K expression is related to the histological type, being more expressed in adenocarcinomas than in squamous cell carcinomas. In a subgroup of cases, hnRNP K immunoreactivity was found not only in the nucleus but also in the cytoplasm of the tumor cells. The cytoplasmic or nuclear accumulation of hnRNP K is highly relevant to determine the role of hnRNP K in a particular cell, since both nuclear and cytoplasmic functions have been assigned to this hnRNP protein. Interestingly, in some lung cancer cell lines we observed a shift from nuclear to nuclear and cytoplasmic localization of hnRNP K when cells were allowed to grow to confluence, suggesting a tight regulation of the localization of the protein in relation to the cell biological status such as cell cycle or microenvironment conditions. These findings are in keeping with the recently reported phosphorylation-dependent cytoplasmic accumulation of hnRNP K in HeLa cells after serum stimulation or constitutive activation of ERK kinase [18].

The observed modifications in the expression of members of the hnRNP family in lung cancer is in agreement with the reported abnormalities in alternative splicing of cancer-related proteins such as BRCA1 [2], cyclin D1 [3], mdm2 [4], FHIT [5], TSG101 [6] or VEGF [7]. One of the best characterized example of a protein with distinct splicing forms in cancer is CD44. CD44 is a surface cell-adhesion molecule expressed by several cell types that can be alternatively spliced to generate many isoforms, some of which are mainly restricted to tumor cells. Alterations in the expression of members of the serine-arginine-rich (SR) family of splicing factors accompany the progressive changes in CD44 alternative splicing in mammary tumorigenesis [45]. hnRNP A1 interacts with regulatory splice elements in CD44 and has been proposed as a decisive part of an oncogene-regulated splice-silencing complex [46,47]. In colon adenocarcinoma, the pattern of CD44 spliced isoforms seems to be determined by the ratio between hnRNP A1 and the SR proteins, with hnRNP A1 antagonizing the SR proteins in the selection of 5′ splicing sites [48]. On the other hand, and in agreement with previous results in myeloid progenitor cells [22], we have observed a relationship between the expression of hnRNP A1 and Bcl-x_L in SCLC cell lines. In BCR/ABL transformed myeloid cells, interference with the hnRNP A1 nucleocytoplasmic trafficking activity results in an enhanced susceptibility to apoptosis a reduced level of Bcl-x_L protein [22]. Bcl-x is a member of the Bcl-2 family with two alternatively spliced forms, Bcl-x_L, capable to protect cell from apoptosis, and Bcl-x_S, a pro-apoptotic variant [49]. Thus, the noticeable increase in the nuclear hnRNP A1 accumulation from normal to hyperplastic, and to malignant lung epithelial tissue observed in our study could have consequences in the splice-choice or the regulation of expression of several mRNAs, such as those for CD44 and Bcl-x proteins.

Although we have focused our study on lung cancer, the RNA processing regulatory proteins may be also relevant in other types of cancer. Preliminary studies indicate that hnRNP A2/B1 is abundantly expressed by breast and pancreatic cancer cell lines and tumors. In both cases, hnRNP A2/B1 has been proposed as a marker for early stages of cancer development [23,24]. hnRNP B1 is also detected in precancerous lesions as well as advanced lesions of human oral squamous cell carcinoma, indicating that hnRNP B1 may be a useful marker for early detection [50]. Surprisingly, a reduction in cytoplasmic staining for hnRNP A2/B1 was observed in thyroid carcinomas when compared with hyperplastic and adenomatous lesions [51]. Altered expression of other alternative splicing regulatory factors has been reported in in vitro models of cellular transformation [52]. hnRNP A1 has been implicated in the genesis of variant pre-mRNA splicing patterns observed between two types of brain tumors, oligodendrogliomas and astrocytomas [21], and a variety of RNA binding proteins have been related to other epithelial [45] and non-epithelial tumors [22,53–55]. Finally, a development of sarcomas has been observed in transgenic mice that overexpress hnRNP D [56], a member of the hnRNP family that has been shown to control the stability of several mRNAs, including that for proto-oncogenes, cytokines, or other signaling molecules.

In conclusion, we have determined the expression of several members of the family of the hnRNP

proteins in normal and malignant lung. Each of these proteins shows different levels of expression and distribution in normal and tumor lung. Our data suggest that critical cellular processes related to RNA maturation may be deregulated in lung carcinogenesis, giving rise to novel tumor-specific protein patterns. An understanding of the regulatory mechanisms of alternative splicing occurring along with neoplastic transformation may help to elucidate some of the causes of abnormal gene expression in lung cancer. More comprehensive studies of the role of the hnRNP proteins in lung carcinogenesis may allow the development of high throughput platforms in which the detailed analysis of the expression of every molecule related to mRNA processing, including hnRNP proteins, may allow the determination of a specific ''mRNA processing machinery profile'' of a particular tumor. The clinical significance of this profiling in terms of early detection, prognosis and patient-based treatment should also be explored in the future.

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