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Report

Differential expression of the early lung cancer detection marker, heterogeneous nuclear ribonucleoprotein-A2/B1 (hnRNP-A2/B1) in normal breast and neoplastic breast cancer

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Summary

Heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP-A2/B1) is highly expressed during critical stages of lung development and carcinogenesis. To determine if the expression of hnRNP-A2/B1 is an informative biomarker in breast carcinogenesis, we analyzed hnRNP-A2/B1 overexpression by immunohistochemistry in archived specimens. Expression was detected in 48/85 (56.5%) primary invasive breast cancers and 7/72 (9.7%) specimens of normal breast tissue. Northern analysis of breast cancer cells also demonstrated higher level of hnRNP-A2/B1 expression compared to normal or transformed breast cells. Expression of hnRNP-A2/B1 in breast cancer cells was decreased by exposure to retinoids coordinately with decreased cell growth. These results warrant further evaluation of hnRNP-A2/B1 as a marker of breast carcinogenesis.

Abbreviations: EGF: epithelial growth factor; 13cRA: 13-*cis* retinoic acid; hnRNP-A2/B1: heterogeneous nuclear ribonucleoprotein A2/B1; SI: Staining Index; 4 HPR: N-(4-hydroxyphenyl) retinamide

Introduction

Breast cancer is the most common malignancy effecting women worldwide [1]. Understanding the initial molecular events leading to the development of breast cancer may provide opportunities for prophylactic intervention, improve our ability to predict breast cancer risk, and lead to improved strategies for early detection. For example, research has focused on identifying cytomorphological changes in breast lesions associated with increased risk for subsequent breast cancer [2–4]. In this report, we explore the frequency of expression of a reported lung cancer marker, heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP-A2/B1) in breast cancer and normal tissue. We have shown that the mouse monoclonal antibody (MoAb) 703D4 binds to this molecule [5]. hnRNPs are members of a family of ribonuclear proteins, which are thought to regulate the shuttling of nascent RNA transcripts between the nucleus and cytoplasm. Recent information suggests other fundamental roles for this important nucleoprotein family [6, 7]. Using immunocytochemical analysis of archival sputum samples, increased expression of this antigen identified 70–90% individuals who later developed lung cancer [8]. Preliminary analysis of two other prospective validation studies also suggests overexpression of hnRNP-A2/B1 in sputum specimens is associated with the identification of individuals who proceed to clinically significant lung cancer [9]. The molecule, hnRNP-A2/B1 is frequently observed in transformed bronchial epithelium, most lung cancer (NSCLC) cells [8] as well as in primary tumors and in some adjacent, histologically-unremarkable bronchial epithelium [10]. Increased expression of hnRNP A2/B1 is also associated with a critical phase of lung development for several mam-

malian systems [11]. We have recently reported that expression of hnRNP-A2/B1 in morphologically normal appearing cells correlated with genetic alterations associated with cancer [12]. We also evaluated this marker for its utility as an intermediate endpoint marker. For example, retinoids have been proposed as chemopreventive agents for breast cancer since they have been found to improve differentiation in many tissues and in mammary and other carcinoma cell lines [13]. In collaboration with investigators from MD Anderson, we found that hnRNP-A2/B1 was modulated *in vitro* in lung cancer cell lines by exposure to 13-*cis* retinoic acid (13cRA). In contrast, the individuals with bronchial metaplasia who received 6 months of 13cRA showed no evidence of modulation of hnRNP-A2/B1 expression *in vivo* [14]. Since breast cancer cell lines were positive in the preliminary immunocytochemistry analysis of MoAb 703D4, the present study was undertaken to determine the expression of hnRNP-A2/B1 in breast cancer cell lines after exposure to a retinoid.

Material and methods

Tissues and immunohistochemistry

A total of 157 specimens of breast tissue were analyzed, comprising 85 cases of primary, invasive breast cancers and 72 cases of normal terminal duct lobular units (TDLUs) breast tissue. The TDLUs are obtained from normal appearing breast tissue obtained from a surgical procedure for benign breast disease. All tissues were fixed in 10% formalin and embedded in paraffin at the Department of Pathology, the University of Texas Health Science Center at San Antonio. No clinical information was available from the individuals who provided those specimens.

Immunohistochemical staining was performed by using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) with previously reported modifications [13]. Five microgram per millilitre of purified mouse antibody was used to identify areas of hnRNP-A2/B1 overexpression. hnRNP-A2/B1 positive human lung cancer tissue was used as positive control. Substitution of the primary antibody by isotopic (IgG 2b) mouse myeloma protein was used as a negative control (Sigma Chemical Co., St. Louis, MO). Levels of hnRNP-A2/B1 expression were scored by two different reviewers (JZ, DCA) using a semiquantitative scoring approach to obtain a staining distribution score and staining intensity score for each specimen. A staining index (SI = distribution score + intensity score) was established for each tissue as previously published (13). The SI ≥ 2 was called positive for hnRNP-A2/B1 expression. Disagreements were resolved after joint review before data analysis.

Cell lines and cell culture

Primary cultures of human mammary epithelial cells (Clonetics, San Diego, CA) and chemically transformed normal breast cell line 184A1 (ATCC) were grown in MEGM (Clonetics, San Diego, CA) at 37°C in 5% CO_2. Chemically transformed normal breast cell line 184B5 (ATCC) was grown in sodium bicarbonate free, MEGM with 10^{-5} M isoproterenol and 5 μg/ml transferrin, at 37°C in 2% CO_2. Transformed breast cell lines, MCF10A (ATCC) [15] and MCF10AT2 (generously provided by Dr. J. Russo), were grown in DMEM/F12 medium (Life Technologies, Rockville, MD) supplemented with 5% horse serum and other factors as described [16, 17]. Breast cancer cell lines MDA-MB-231, T-47D, SK-BR-3 and MCF7 (ATCC) were grown in Improved MEM Zinc Optimum medium (Life Technologies, Rockville, MD) supplemented with 5% fetal calf serum. The cells were cultured in chamber slides, washed, fixed in formalin for 10 min, and analyzed by immunocytochemistry.

Northern blot assays

The northern blot assay was performed essentially as described previously [5]. A ^{32}P-labelled 1.1 kb human hnRNP A2 cDNA fragment, which was generated by RT-PCR amplification with hnRNP primers on mRNA from NSCLC cell line NCI-H720, encompassing the hnRNP A2 coding region [11] and 1.4 kb human hnRNP K cDNA (a generous gift of Dr. David Levens, Laboratory of Pathology, NCI/NIH, Bethesda, MD) were used as hybridization probes. The relative amount of mRNA transcripts were analyzed by using a PhosphorImager and Personal Densitometer SI (Molecular Dynamics, CA).

In vitro *hnRNPA2/B1 modulation by 13-*cis *retinoic acid*

Human breast cancer cell lines T47D and MB231 were seeded in 6-well plates at 1–2 $\times$ 10^5 cells/ml in serum free hormonally defined medium [18]. 13-*cis* retinoic acid (13cRA) (Sigma Chemical Co., St. Louis,

MO) was added at final concentrations of 0.5–2.0 μM. After 24 h incubation, cytospins were prepared and fixed in 10% buffered formalin for 10 min, and immunocytochemistry was performed using a HistoStain Kit (Zymed Laboratories Inc., San Francisco, CA), following the manufacturer's protocol.

To better estimate the effect of RA on the breast cancer cell lines, confocal microscopy was performed. After treatment, cytospins were fixed in Bouin's fluid (Sigma, St. Louis, MO) for 10 min and exposed to the monoclonal antibody 703D4 overnight at 4°C, as described above. The next day, the slides were incubated for 1 h in Bodipy goat anti mouse (Molecular Probes, Eugene, OR) 1:200, followed by 10 min in DAPI (Molecular Probes, Eugene, OR). After thorough washes, the slides were mounted in SlowFade solution (Molecular Probes, Eugene, OR) and observed with a Zeiss Laser Scanning Microscope 510, equipped with four lasers.

Statistical analysis

Fisher's exact test (2-tail) was used to test the difference of mean SI between two groups, primary invasive breast cancer and normal breast terminal duct lobular units (TDLUs).

Results

Immunohistochemistral analysis of hnRNP-A2/B1 overexpression in primary invasive breast cancer vs. normal breast tissue

The results of immunohistochemistry of 85 cases of primary invasive breast cancer and 72 specimens of normal breast are summarized in Table 1. hnRNP-A2/B1 overexpression was found in primary invasive breast cancer specimens in 48 out of the 85 (56.5%) cases. The pattern of hnRNP-A2/B1 expression was variable in regard to both intensity and distribution. Both diffuse cytoplasmic and granular cytoplasmic staining were observed in the positive cases (Figure 1A & B). In contrast, only seven out of 72 (9.7%) normal breast samples were positive for hnRNP-A2/B1 with faint cytoplasmic immunostaining of the TDLUs. When we analyzed the mean SI of hnRNP-A2/B1 expression in the two groups by Fisher's exact test, significantly increased levels of hnRNP-A2/B1 expression were found in the primary invasive breast cancer group ($p < 0.001$).

Table 1. hnRNP A2/B1 expression in normal breast or invasive breast cancer tissue

Case	Positive (%)	Negative (%)	Mean SI ± S.E.M.* (Number of specimens)
Normal breast	7 (9.7)	65 (90.3)	0.26+0.1 (72)
Breast cancer	48 (56.5)	37 (43.5)	1.98+0.21 (85)
Total	55	102	(157)

* P value of the mean SI between normal and breast cancer was < 0.001.

hnRNP-A2/B1 expression in breast cell lines

By immunocytochemistry analysis, the *in vitro* expression of hnRNP-A2/B1 varied but was observable in primary invasive cultures of normal human mammary epithelial cells, transformed breast cell lines and breast cancer cell lines (Figure 1C-F). Normal human mammary epithelial cells, transformed cell lines and the other two breast cancer cell lines showed diffuse cytoplasmic staining (Figure 1C, 1E, 1F). In contrast, the breast cancer cell line, MDA-MB-231 showed granular cytoplasmic expession (1D). No significant differences were observed by calculating the SI from each cell line. To evaluate the relative expression of hnRNP mRNA, we did parallel northern blotting. Digitized signal intensity of the northern blot was adjusted for loading differences by quantitation of the 28S rRNA band. As demonstrated in Figure 2, the level of mRNA for hnRNPA2/B1 was generally higher in the breast tumor cell lines than in normal and transformed breast cell lines. The mRNA expression in two normal primary breast cell cultures for hnRNP-A2/B1 was more abundant than in the chemically transformed cell lines (184A1 and 184B5) than in the spontaneously immortalized line, MCF10A. In addition, c-Ha-ras transformed cell line MCF10AT2 has higher hnRNP-A2/B1 expression than its parent non-tumorigenic spontaneously immortalized line, MCF10A (Figure 2).

The same blots were probed for the related nucleoprotein, hnRNP K. The level of hnRNP-K mRNA was more uniformly expressed between the different groups than hnRNP- A2/B1 (Figure 2).

*hnRNP-A2/B1 modulation by 13-*cis *retinoic acid*

We evaluated the effects of *in vitro* 13cRA exposure on breast cancer cells using retinoid doses known to be inhibitory in a semi-automated proliferation assay

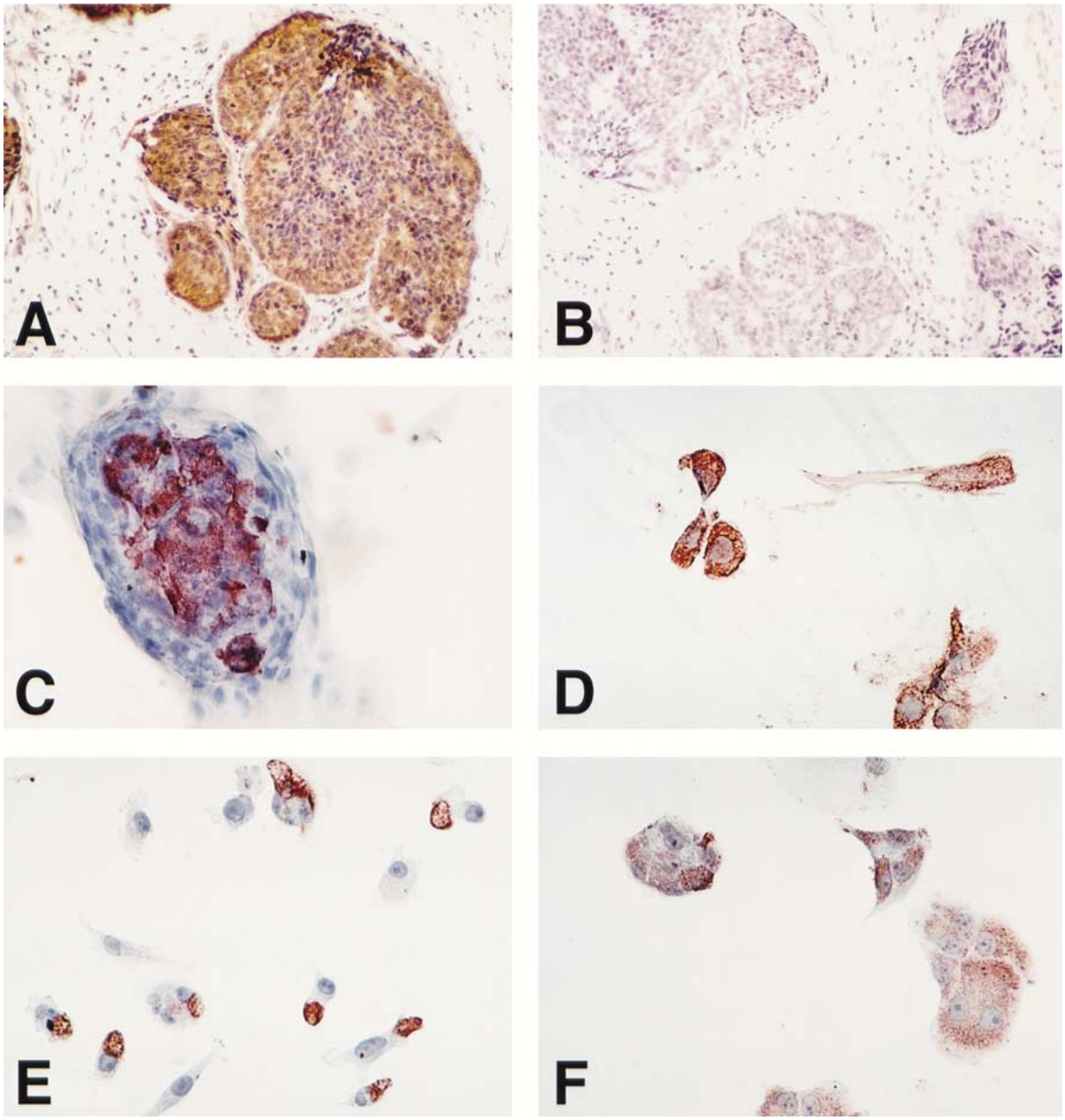

Figure 1. Immunocytochemical staining pattern for hnRNP A2/B1 in clinical pathological specimens and in tumor cell lines of the breast. (A) Paraffin section of a primary breast cancer showing diffuse cytoplasmic staining in the tumor cells. (B) Serial section showing the specificity of the staining, which was confirmed by the negative signal generated by an isotypic monoclonal antibody. (C) Normal human mammary epithelium cell, 184A1. (D) Transformed cell line, MCF10A (E) Breast cancer cell line, MDA-MB-231. (F) Breast cancer cell line, T-47D.

[18]. Using immunocytochemistry to study the modulation of hnRNP-A2/B1 expression after exposure to 13cRA or 4-HPR, we observed a marked decrease of hnRNP-A2/B1 expression in the cell lines, MDA-MB-231, MCF7 and T-47D (Figure 3). The decrease in immunoreactivity in the retinoid treated cells was due to reduction in both antigen distribution and intensity for all tested cell lines. In addition, we observed that treatment with 13cRA induced consistent translocation of hnRNP-A2/B1 immunoreactivity from the nucleus to the cytoplasm in MDA-MB-231 (Figure 4).

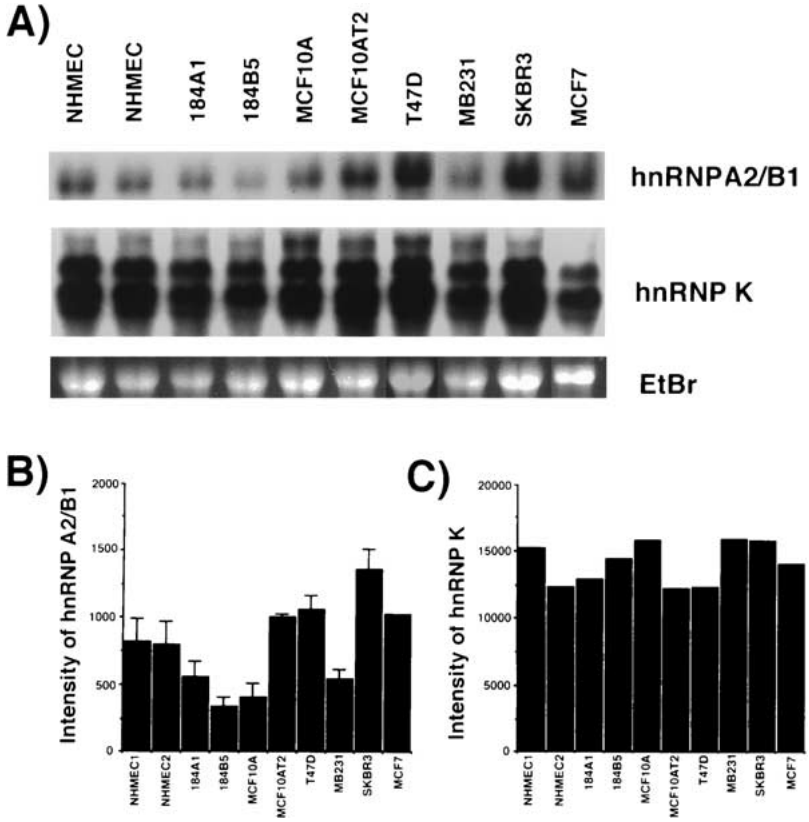

Figure 2. (A) Northern blot analysis of the cell lines studied in this article. The same blot was sequentially proved with hnRNP A2/B1 and hnRNP K. The ethidium bromide image of the 28S rRNA was measured for normalizing loading efficiency. (B,C) Image analyzer quantitations of the northern blot bands is shown after correction with the rRNA values, and are represented for both hnRNP A2/B1 (B) and hnRNP K (C).

Discussion

Better understanding of the molecular events of carcinogenesis may help improve outcome for this disease by indentifying markers allowing early detection. Identification of breast cancer may be possible using signals that are differentially expressed in other epithelial cancer. Increased expression of hnRNP-A2/B1 has been reported as an early lung cancer detection marker which can detect 70–90% of true positive lung cancer [8, 9]. This molecule present a tightly regulated expression during fetal development, and has restricted expression in normal adult tissue [11]. In an effort to determine the properties of hnRNP-A2/B1 as a general marker of epithelial carcinogenesis, we have evaluated the potential clinical relevance of hnRNP-A2/B1 expression status in breast cancer, in both clinical samples and cultured cell lines.

This study reveals that hnRNP-A2/B1 was more frequently expressed in primary invasive breast cancer (56%) than in normal breast tissue from individuals free of cancer (9.7%). This finding is similar to our earlier studies on lung cancer [5, 10, 11]. The presence of positive cells in morphologically normal tissue is in agreement with observations in lung tissues when hnRNP-A2/B1 positively correlated with increased genetic instability that made these histologically unremarkable cells good candidates for premalignant cells [12]. In contrast, there was no significant difference in hnRNP-A2/B1 protein expression in cultured normal breast versus tumor breast cells by immunohistochemical detection. The significance of the finding of hnRNPA2/B1 expression in the two tested specimens of normal cultured breast epithelial cells may be related to the particular phenotype exhibited by these cells in culture. The higher level of expression for hnRNP-A2/B1 mRNA seen in the two normal primary cultured cells as compared with non-tumorigenic transformed breast cell lines suggests that the expression level of hnRNP-A2/B1 may also relate to proliferation status as we have previously reported [5]. The general expression levels

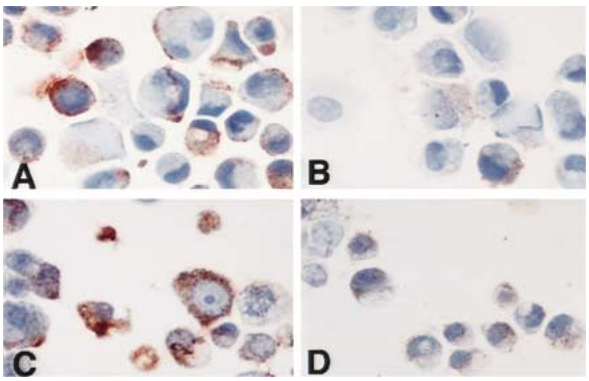

Figure 3.

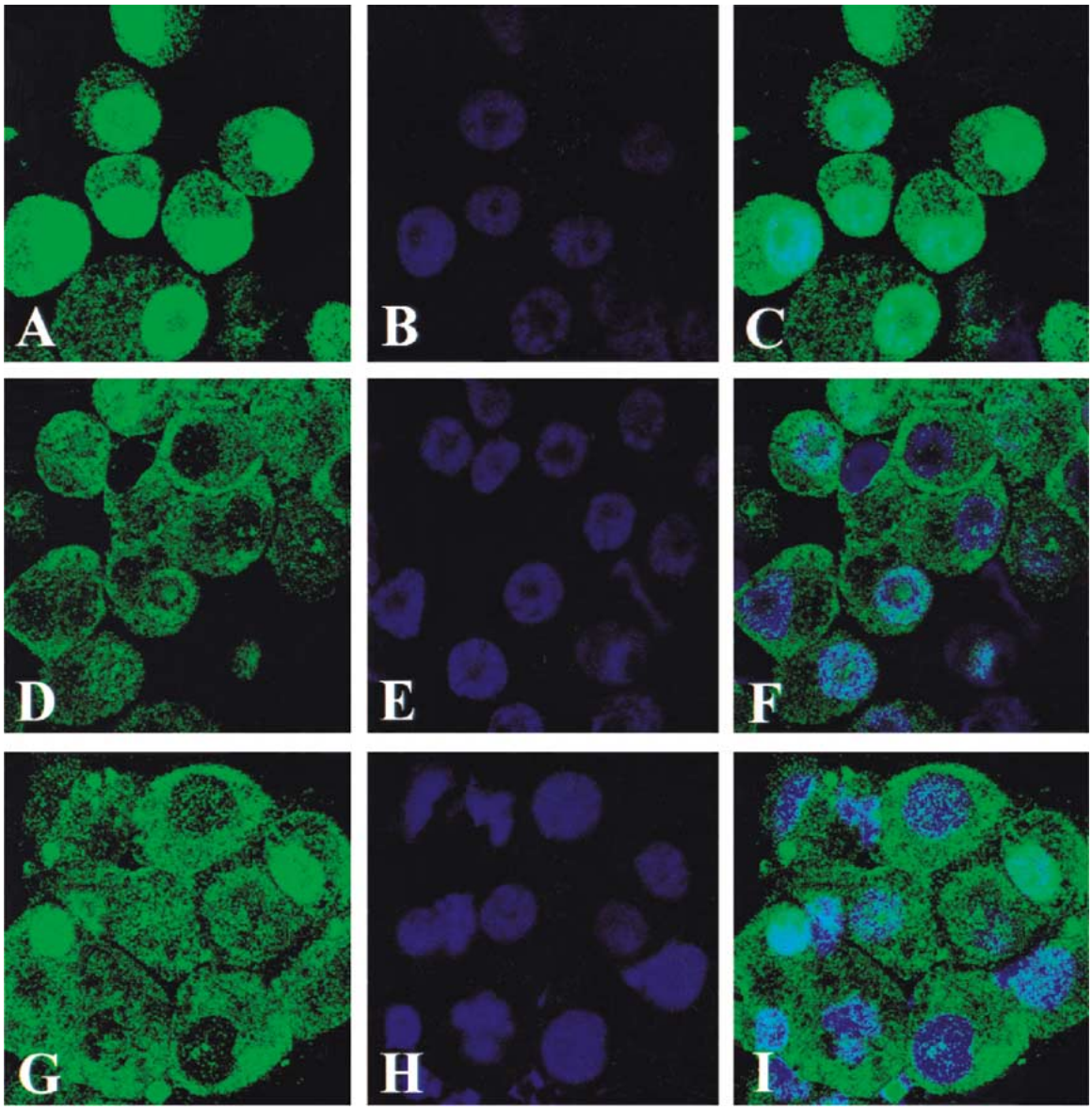

Figure 4.

Figure 3. Effect of the retinoid treatment on the staining pattern of hnRNP A2/B1 in cultured cells. Cell lines MB231 (A,B) and T-47D (C,D) are shown under normal conditions (A,C) and after treatment with 2 μM 13cRA (B,D).

for hnRNP-A2/B1 mRNA are higher in the tumor cell lines indicating an overexpression in these cells as compared to normal and transformed breast cell lines. Other studies have shown that upregulation of hnRNP is associated with cell proliferation [19, 20] so the levels of hnRNP-A2/B1 *in vitro* may not be comparable to those of the cells growing on an intact basement membrane. Our original data on lung cells in culture showed that transformed and cancer cells lack the ability to down-regulate hnRNP-A2/B1 expression when growth arrested by serum deprivation. A higher level of hnRNP-A2/B1 expression in transformed and lung cancer cell lines compared to normal primary bronchial epithelial cell cultures and the overexpression of hnRNP-A2/B1 in lung epithelial transformed and tumor cells is not subject to proliferation-dependent control [5]. More recently, we have shown that hnRNP A2/B1 over expression in respiratory tissue is highly correlated with the presence of other markers of genetic instability independent of the state of differentiation of the tissue [12]. In this study the highest expression level of hnRNP-A2/B1 was found in breast cancer cell lines, as well as the c-Ha-ras transformed cell line, MCF10AT2. In light of the frequent expression of hnRNP-A2/B1 in primary invasive breast cancer compared to benign tissue, further evaluation of overexpression of hnRNP-A2/B1 as a breast cancer early detection marker in a prospective analysis to determine its clinical utility is also warranted.

In the current study, we observed down regulation of hnRNP-A2/B1 protein expression in two breast cancer cell lines treated with 0.5–2 μM 13cRA. Based on these pilot findings a larger series of experiments is under way to determine whether hnRNP-A2/B1 could be used as an intermediate endpoint marker for chemoprevention studies. Furthermore, we have observed that when hnRNP A2/B1 is expressed in the nucleus that retinoids modulate the translocation of hnRNP-A2/B1 from the nucleus to the cytoplasm. This finding suggests that this ribonucleoprotein may relate with the differentiation status of breast cancer cells and this issue merits further investigation to clarify the clinical utility of this finding.

Figure 4. Confocal microscopy of cell line MDA-MB-231 stained for hnRNP A2/B1 (green, A,D,G) and counterstained with DAPI (blue, B,E,H). The third column (C,F,I) represents the combination of the previous images. Untreated cells demonstrate strong nuclear expression of hnRNP A2/B1 (A-C), but after treatment with 13cRA for 24 h (D-F) nuclear staining totally disappears. Treatment with 1 μM 4-HPR (G-I) resulted in a partial abolition of nuclear staining. Note the high incidence of apoptotic bodies in H.

We conclude that hnRNP-A2/B1 expression occurs in most primary invasive breast cancer compared with 10% of normal breast tissue. Both mRNA and protein of hnRNP-A2/B1 were expressed in normal human primary mammary epithelial cultures, transformed breast cell lines, and primary tumor cell lines. Increased mRNA expression was found in the breast tumor cell lines. The expression of hnRNP-A2/B1 in breast cancer cells was modulated by the exposure to 13-*cis* retinoic acid (13 cRA) or 4 HPR *in vitro*. Further experiments need to be carried out to determine the precise role of hnRNP-A2/B1 in breast carcinogenesis.

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