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Nucleolar Antigens and Autoantibodies in Hepatocellular Carcinoma and Other Malignancies

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Patients with hepatocellular carcinoma (HCC), gastrointestinal, lung, and ovarian cancers were shown to have autoantibodies to nuclear and nucleolar antigens as detected by immunofluorescence on cell substrates. The frequency of antinuclear antibodies (ANAs) was significantly higher ($P < 0.001$) in patients with HCC (57/184 = 31%) than in patients with chronic hepatitis or liver cirrhosis (25/187 = 13%). Although a range of fluorescence patterns was observed, a higher percentage of nucleolar fluorescence was detected in HCC, and three of these nucleolar antigens were identified. They were NOR-90, nucleolus organizer region doublet polypeptides of 93 and 89 kDa involved in RNA polymerase I transcription; fibrillarin, a 34 kDa protein of the nucleolar U3 ribonucleoprotein particle which is engaged in preribosomal RNA processing; and nucleophosmin/protein B23, a 37 kDa polypeptide which is associated with ribosome maturation and cellular proliferation. All these antigens are nucleolar components that are engaged in some aspect of ribosome biosynthesis. Since autoantibodies to these nucleolar antigens have also been found in systemic autoimmune diseases, they do not represent autoimmune reactions unique to cancer but might reflect reaction pathways related to immune responses that are antigen-driven. The ANA response in HCC appears to be dynamic reactions to this antigen-drive since some patients with chronic liver disease showed seroconversion to ANA positivity, marked increase in titer and/or change in antibody specificity preceding or coincident with clinical detection of HCC. These changes in ANA showed a close temporal relationship with transformation from long-established chronic liver disease to HCC. (Am J Pathol 1992, 140:859–870)

Autoimmune phenomena manifested as antibodies to cellular components have been described in cancer patients. They include antinuclear antibodies (ANA) and antibodies to cytoplasmic antigens.[1–12] Autoantibodies have been detected in patients with leukemias; malignant melanomas; lung, breast, gastrointestinal, gynecologic, nasopharyngeal, and prostate carcinomas; hepatocellular carcinoma (HCC); and neurologic paraneoplastic syndromes. In sera of patients with malignant melanomas, autoantibodies reactive with nuclear, nucleolar and cytoplasmic antigens were found in 90% of the cases with an indirect immunofluorescence technique using several cell lines as substrate,[4] whereas 74% were positive by Western blotting.[13] In patients with HCC, the prevalence of ANA was reported to be 10–32%.[11,14,15] An antigen in retinal rod and cone cells has been identified in cancer-associated retinopathy[16] and cDNA clones encoding a protein antigen recognized by antineuronal cell antibodies in paraneoplastic cerebellar degeneration have been isolated.[17,18]

Autoantibodies, especially ANAs, are used as serologic markers in systemic lupus erythematosus and other systemic autoimmune diseases such as scleroderma, Sjögren's syndrome, and dermatomyositis. Many of the antigens reactive with antibodies in these diseases have been structurally and functionally defined[19] and were found to be components of larger subcellular particles that are involved in important or essential cell functions such as DNA replication, DNA transcription, and RNA processing. Epitopes recognized by autoantibodies are often active regions or functional sites of these complex particles. From these and other data, it was postulated

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that autoimmunity in these diseases is related to an antigen-driven mechanism.[19]

The significance of the previously reported autoantibody responses to self antigens in malignancies is unclear and there is little information regarding the identity of many of the antigens. Characterizing the structure and function of intracellular antigens in malignancy may provide insights into whether autoantibodies are being generated against antigens unique to cancer or are immune responses to proteins whose functions are abnormally expressed or regulated in malignant cells. In this study, we have focused on autoantibodies to the nucleolus in HCC and other malignancies and identified three nucleolar antigens: nucleophosmin/protein B23, nucleolus organizer region (NOR) proteins of 93 and 89 kDa (called NOR-90), and fibrillarin, a component of the U3 ribonucleoprotein (RNP) particle. These nucleolar components are associated with ribosome biosynthesis[20–22] and mitogenesis,[23] functions that are integral parts of processes related to cellular proliferation.

Materials and Methods

Patients

Patients were treated in the clinics and hospital of Shinshu University School of Medicine in Matsumoto, Japan, and were on the service of the Second Department of Internal Medicine. Sera were obtained from 184 patients (153 men, 31 women, mean age, 59.0 years) with HCC (71 associated with hepatitis B, 106 with non-A non-B hepatitis, 6 with alcoholic liver disease, and 1 with autoimmune hepatitis), 210 patients with alimentary tract cancers (14 with esophageal, 50 with stomach, 50 with pancreatic, 46 with bile duct and 50 with colon cancer), 37 patients with lung cancer, and 2 patients with ovarian cancer. These serum samples were collected before cancer therapy was initiated so that the immune alterations observed were not influenced by therapy. Serial serum samples were available from four patients with HCC which showed positive ANA and could be analyzed before and after diagnosis of HCC. As controls, sera were obtained from 123 patients with chronic hepatitis and 64 patients with liver cirrhosis (69 with hepatitis B, 114 with non-A non-B hepatitis, and 4 with alcoholic cirrhosis), and 229 normal healthy Japanese adults (mean age, 62.1 years; male/female ratio, 2.3) (Table 1).

Reference Antibodies

The antibody to NOR-90 was a gift from Dr. M.J. Fritzler (University of Calgary, Calgary, Alberta) and had been used in earlier studies to identify the antigen.[24] The antibodies specific for fibrillarin were previously characterized.[25–27] Monoclonal antibody to protein B23 was a gift of Dr. P.K. Chan (Baylor College of Medicine, Houston, TX), and its specificity was described previously.[28]

Cell Lines

Human HEp-2 epithelial cells, rat kangaroo PtK2 kidney epithelial cells, rat Novikoff hepatoma cells, and Indian muntjac skin fibroblast cells were obtained from the American Type Culture Collection, Rockville, MD, and cultured according to the specifications described for each cell line.

Immunofluorescence

Identification of ANAs was performed using methanol- and acetone-fixed commercial HEp-2 cell slides (Bion Enterprises, Ltd., Park Ridge, IL). This and other cell types were also prepared in this laboratory, grown on

Table 1. *Antinuclear Antibodies in Hepatocellular Carcinoma, Chronic Liver Disease, and Other Malignancies*

Category	Number of patients	Antinuclear antibodies					
		Total positive (%)	H*	S	N	MSA	C
Hepatocellular carcinoma	184	57 (31)	10	32	12	2	1
Chronic hepatitis or cirrhosis	187	25 (13)	13	11	1	—	—
Alimentary tract cancer	210	20 (9)	4	12	2	1	1
Lung cancer	37	5 (13)	—	4	1	—	—
Ovarian cancer	2	1	—	—	1	—	—
Normal controls	229	11 (5)	2	8	1	—	—

* Patterns of nuclear fluorescence.
H = homogeneous, S = speckled, N = nucleolar, MSA = mitotic spindle apparatus, C = centromere.
Indirect immunofluorescence on HEp-2 cells.

glass coverslips, fixed with either 2% formaldehyde (Polysciences Inc., Warrington, PA) for 20 minutes at room temperature or with 100% methanol (Fisher Scientific Co., Fair Lawn, NJ) for 5 minutes at −20°C, and then permeabilized with 100% acetone (Fisher Scientific Co.) for 3 minutes at −20°C. The presence of ANA was detected by indirect immunofluorescence. As a second antibody, FITC-conjugated goat anti-human IgG (Caltag Laboratories, SF, CA) was applied. A titer of more than 1:40 was interpreted as positive. For double-label immunofluorescence, mouse monoclonal antibody was mixed with patient serum and fluorescence was detected with both FITC-conjugated goat anti-human IgG and rhodamine-conjugated goat anti-mouse IgG (Caltag Laboratories).

Nucleolus Segregation

Segregation of PtK2 nucleoli into fibrillar and granular components was induced by the addition of 0.2 μg/ml actinomycin D to the culture medium for 4 hours,[26] after which cells were processed for immunofluorescence.

Chromosomal Spreads and Silver Staining

To prepare chromosomal spreads, mitotic cells were collected by mild trypsinization of actively growing Indian muntjac cells that were treated with Colcemid (0.01 μg/ml) for 6 hours before harvest. Chromosome spreads were prepared by the method of Merry et al[29] and then processed as described for immunofluorescence. After the immunofluorescence procedure, chromosome spreads were counterstained with ethidium bromide (1 μg/ml) for 5 minutes and rinsed in water for 5 minutes. To stain the NORs of chromosomes with silver, the procedure of Ploton et al[30] was used with slight modification. After immunofluorescence, the coverglass was gently removed and rinsed with deionized water. The staining solution was prepared by mixing one volume of 2% gelatin in 1% aqueous formic acid and two volumes of 50% aqueous silver nitrate solution. This was poured over the chromosomal spread and left for 60 minutes at room temperature. The silver colloid was then washed off with deionized water and allowed to air dry. The specimens were dehydrated with xylene and mounted in Permount (Fisher Scientific Co.).

Western Blotting

HEp-2 cell nuclei and rat Novikoff hepatoma cell nuclei were isolated from cultured cells as described by Muramatsu et al[31] using aprotinin (1 μg/ml), leupeptin (1 μg/ml), pepstatin A (1 μg/ml), and phenylmethyl-sulfonyl fluoride (1 mM) as protease inhibitors. Isolated nuclei were solubilized in Laemmli's sample buffer.[32] The nuclear extract was first subjected to electrophoresis on 15% or 7.5% SDS-polyacrylamide gels and then electrophoretically transferred to nitrocellulose paper. The nitrocellulose paper strips were incubated for 1 hour at room temperature with a 1:100 dilution of serum using PBS containing 0.5% Tween-20 and 3% nonfat dried milk as diluent after they had been preblocked with PBS containing 3% nonfat dried milk for 30 minutes at room temperature. Nitrocellulose strips were then reacted with ^{125}I-protein A (ICN Biomedicals Inc., Irvine, CA) for 30 minutes at room temperature. To detect reactivities of mouse monoclonal antibodies, ^{125}I-goat anti-mouse IgG (ICN Biomedicals) was used for 60 minutes instead of ^{125}I-protein A. X-OMAT AR films (Eastman Kodak Co., Rochester, NY) were exposed to the nitrocellulose strips at −70°C overnight.

Two-dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was performed by the method of O'Farrell.[33] Whole nuclei were suspended in urea solubilization buffer containing 9.5 M urea, 2% (w/v) NP-40, 2% Ampholine (pH range 3.5–10) (Pharmacia-LKB, Piscataway, NJ) and 5% 2-mercaptoethanol. Nonequilibrium pH gradient electrophoresis (NEPHGE) was employed as the first dimension using broad range pH (3.5–10) Ampholine, then proteins were further separated on 12.5% SDS-polyacrylamide gels.

Immunoaffinity Purification of Antibodies

Antibodies were affinity-purified from nitrocellulose strips by a modified procedure of Burke et al.[34] Three percent nonfat dried milk and 0.5% Tween-20 were used instead of 10% (v/v) new born calf serum and 0.2% Triton X-100. Nitrocellulose strips were washed with phosphate-buffered saline (PBS) containing 0.5% Tween-20, followed by PBS. Bound antibodies were eluted with 0.1 M glycine-HCl (pH 2.5) containing 0.1% bovine serum albumin and immediately neutralized by addition of 1 M Tris HCl (pH 8.7). Eluted antibodies were concentrated with a microconcentrator (Amicon, Danvers, MA). Affinity-purified antibodies were used without dilution for immunofluorescence or diluted 1:5 for Western blotting.

Statistical Analysis

Data were compared using the chi-square test with Yates' correction.

Results

A total of 433 cancer patients were analyzed for ANAs by indirect immunofluorescence using HEp-2 cells as substrate (Table 1). Patients with HCC had a higher frequency of ANAs (31%) compared with healthy subjects (5%) and patients with chronic hepatitis or cirrhosis (13%) ($P < 0.001$). The frequency was 9% in alimentary tract cancers including stomach, esophageal, bile duct, and colon cancers and 13% in lung cancer and although higher than the frequency of 5% in sex and age-matched healthy Japanese adults, it did not reach statistical significance ($P < 0.1$). A variety of nuclear staining patterns could be distinguished including speckled and homogeneous nucleoplasmic staining, nucleolar staining, and patterns related to the mitotic spindle apparatus and centromeric regions of chromosomes. Antinucleolar and speckled antinucleoplasmic antibodies were observed more frequently in HCC than in chronic hepatitis or cirrhosis ($P < 0.01$).

Further studies were focused on antinucleolar antibodies. In general, the types of nucleolar fluorescence observed could be classified into clumpy, speckled, and homogeneous as described previously[35] and depicted in Figure 1. Serum W.K., from a patient with HCC, showed a clumpy type of nucleolar staining on HEp-2 cells (Figure 1A) and a readily distinguishable feature was staining of the periphery of chromosomes in mitotic cells (arrowhead). Serum I.I., also from a patient with HCC (Figure 1B), demonstrated a speckled type of nucleolar staining associated with lower intensity finely speckled nucleoplasmic staining. In HEp-2 cells undergoing mitosis (arrowhead), staining of several pairs of dots could be observed, and as described in more detail later, these represented chromosomal NORs. A third pattern of nucleolar staining was demonstrated by patient W.S., also with HCC (Figure 1C), showing a homogeneous pattern of nucleolar staining. In mitotic cells (not represented in this photomicrograph) the staining was dispersed in the cytoplasm.

Ten of the 18 sera with antinucleolar antibodies had titers of 1:160 to 1:5120 and these higher titered sera were selected for further analysis by Western blotting using HEp-2 cell nuclear extracts as antigen. These ten sera were obtained from eight patients with HCC, a patient with lung cancer and a patient with ovarian cancer.

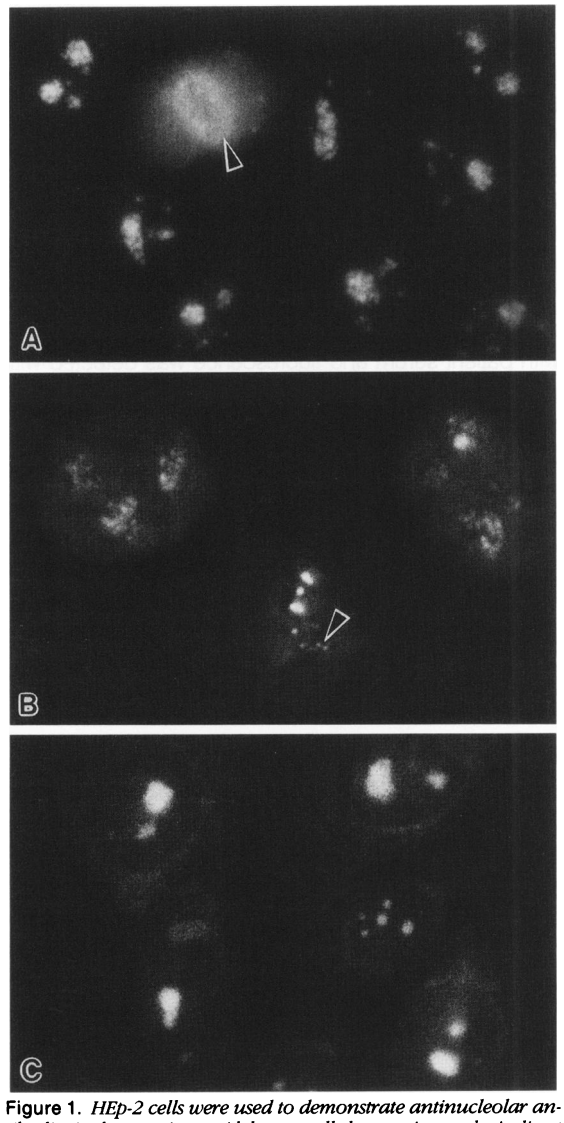

Figure 1. *HEp-2 cells were used to demonstrate antinucleolar antibodies in three patients with hepatocellular carcinoma by indirect immunofluorescence.* **A:** *Patient W.K., with autoantibody to fibrillarin, shows a clumpy nucleolar pattern with staining around chromosomes in a metaphase cell (arrowhead).* **B:** *Patient I.I., with autoantibody to NOR-90, shows speckled nucleolar staining with smaller condensed dots depicting staining of NORs in a mitotic cell (arrowhead).* **C:** *Homogeneous nucleolar staining demonstrated by patient W.S., with autoantibody to B23. Staining of the nuclear membrane and cytoplasm may be related to other autoantibodies present in this serum, magnification ×800.*

Previous experience had shown that sera that had titers of 1:80 and lower in indirect immunofluorescence were nonreactive in Western blots. All of the ten sera with high-titered antinucleolar autoantibodies were positive in Western blots and several reactive bands were detected, ranging from apparent molecular weights of 120, 99, 93/89 doublet, to 37 and 34 kDa (Figure 2A). Serum I.I. blotted a "doublet" migrating at 93 and 89 kDa. Serum W.K. blotted a 34 kDa band and at least three other higher

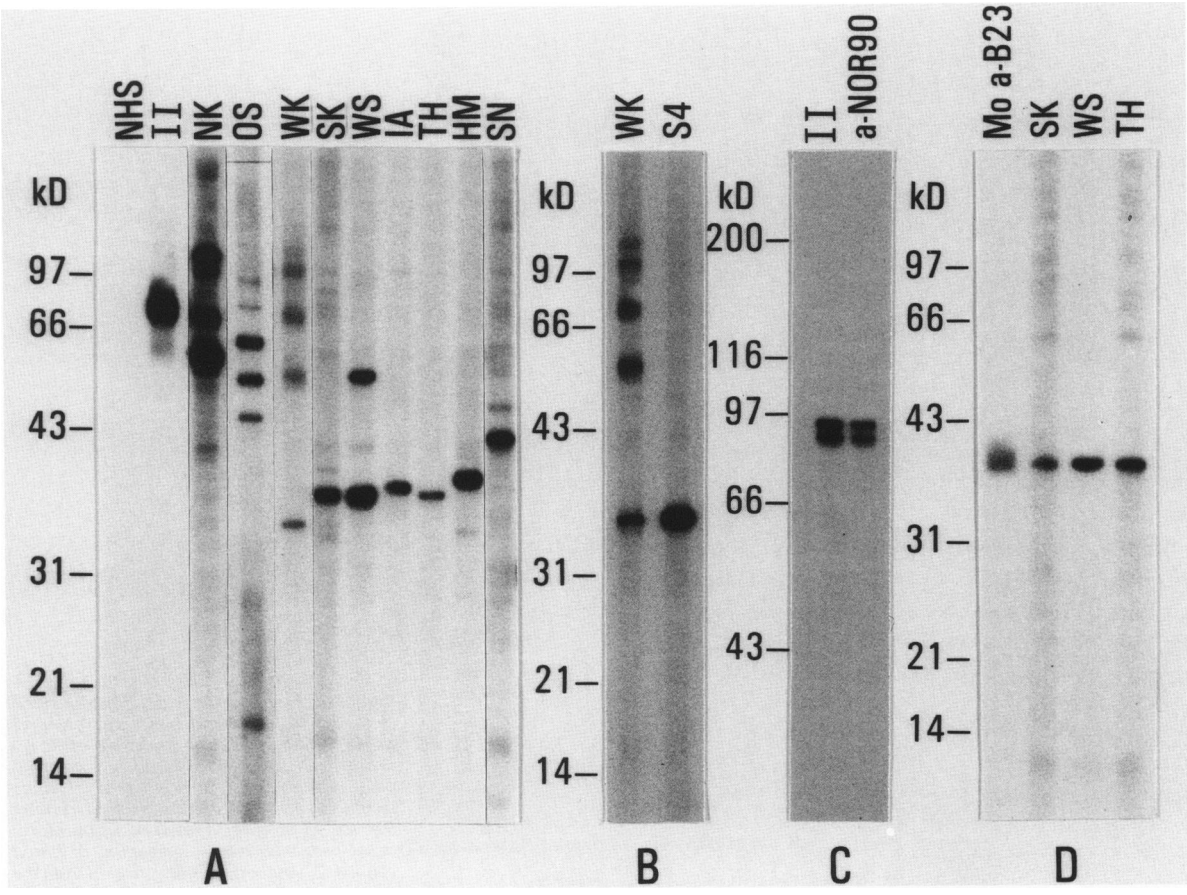

Figure 2. *Western blotting of ten cancer sera* **(A)** *that were selected because they contained antinucleolar antibodies of high titer. Lane I.I. shows blotting of a doublet of 93 and 89 kDa (the doublet appears as a fused blot in this overexposed autoradiograph). Lane W.K. shows blotting of a 34 kDa band and other higher molecular weight bands. Lanes S.K., W.S., and T.H. show blotting of a band at 37 kDa;* **(B)** *shows that the 34 kDa band blotted by W.K. serum migrates with the same mobility as prototype serum S4 which is monospecific for fibrillarin;* **(C)** *shows the comigration of bands blotted by serum I.I. and prototype serum containing anti-NOR-90;* **(D)** *shows the comigration of bands blotted by S.K., W.S., and T.H. with the band reactive with monoclonal antibody to B23. HEp-2 cell nuclear extract was used as antigen in* **(A)**, **(B)**, *and* **(C)** *and rat Novikoff hepatoma cell nuclear extract in* **(D)**. *Numbers in margins refer to molecular masses of protein markers.*

molecular weight bands. Three sera, S.K., W.S., and T.H. blotted a common 37 kDa band, although they showed other reactivities as well. The assumption was made that the reactive antigens were nucleolar in origin, based on the intentional selection of higher-titered antinucleolar antibody sera for this study. If this assumption was justified, then the 34, 37, and doublet 93/89 kDa antigens could be related to certain nucleolar protein antigens that had previously been identified and characterized using autoantibodies from patients with scleroderma and other autoimmune disease.[24–26,36] The subsequent studies determined whether the immunologic and biochemical properties of the antigens reactive with sera from patients with carcinoma were identical to those identified in autoimmune diseases. If they were found to be identical, important considerations would be raised regarding the nature of immunogenic stimuli initiating autoantibody formation.

Our first approach to preliminary identification of these antigens was to run the cancer sera side by side with prototype sera from patients with lupus and other systemic autoimmune diseases. Figure 2B shows that serum W.K. blotted a 34 kDa protein that migrated in the same position as a protein recognized by prototype serum S4 from a patient with scleroderma and known to contain antibody to fibrillarin, a 34 kDa protein of the nucleolus.[25] Serum I.I. (Figure 2C) blotted a 93/89 kDa doublet in the same position as a prototype serum to NOR-90[24] and sera S.K., W.S., and T.H. (Figure 2D) showed reactivity with a band at 37 kDa recognized by a monoclonal antibody to nucleolar protein B23.[28]

Some of the five sera contained antibodies of other specificities as demonstrated by Western blotting, and the possibility that blotting of the putative antigens might have been due to crossreacting antibodies was ruled out. Affinity-purified antibody from the 34 kDa band of

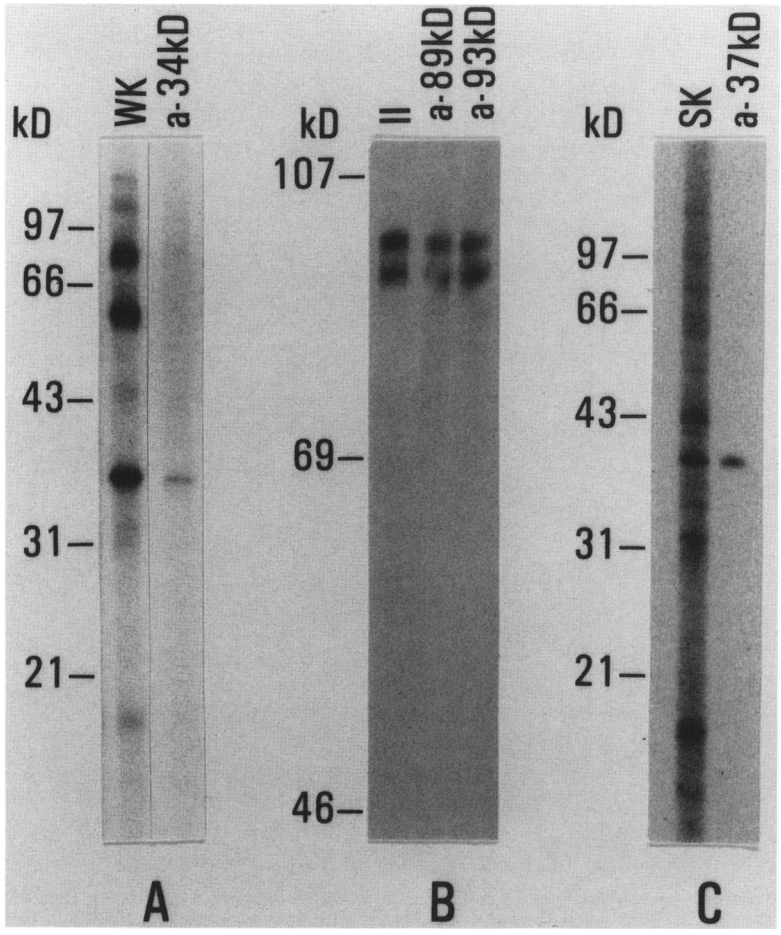

Figure 3. A: *Antibody was affinity-purified from the 34 kDa band of serum W.K. and used in Western blotting to show specific reaction with the 34 kDa band but not with other reactive bands demonstrated by whole serum.* **B:** *Antibody affinity-purified from either the 93 or 89 kDa band of serum I.I. reacted with both bands, suggesting a shared epitope.* **C:** *Antibody affinity-purified from the 37 kDa band of S.K. reacted only with the 37 kDa band. HEp-2 cell nuclear extract was used as antigen.*

W.K. reacted only with the 34 kDa band of HEp-2 total nuclear extracts (Figure 3A) and was nonreactive with other bands that were detected by the whole serum. Affinity-purified antibody from either the 93 or 89 kDa band of serum I.I. (Figure 3B) was observed to blot both the 93 and 89 kDa bands. Affinity purification from either band could have resulted in cross contamination with antibody from the adjacent band, but repeated studies gave the same results, suggesting that there might be shared epitope(s) between the two proteins. Finally, affinity-purified antibody from the 37 kDa band of serum S.K. (Figure 3C), W.S., or T.H. also reacted monospecifically with a 37 kDa protein of HEp-2 cell extract.

The affinity-purified antibodies from the 34 kDa band, the 93/89 kDa doublet, and the 37 kDa band were also examined in indirect immunofluorescence on HEp-2 cells. If these affinity-purified antibodies were reacting with the putative nucleolar antigens, they would be expected to demonstrate characteristic immunofluorescence localization as had been established in previous studies.[24,28,37] Affinity-purified antibody from the 34 kDa band of serum W.K. gave a clumpy nucleolar staining pattern compatible with fibrillarin (Figure 4A), antibody from serum I.I. gave a speckled nucleolar staining and reacted with chromosomal NORs as shown in an anaphase cell (Figure 4B), and antibody from serum S.K., W.S., or T.H. reacted to give a diffuse nucleolar staining as reported previously for protein B23 (Figure 4C).

It has been shown that when tissue culture cells such as PtK2 are treated with actinomycin D, the nucleolus undergoes structural changes that segregate nucleolar components into discrete fibrillar and granular regions.[38] The 34 kDa protein fibrillarin has been shown by immu-

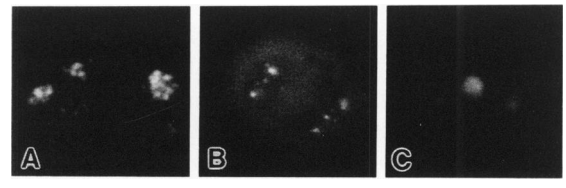

Figure 4. *Immunofluorescence with affinity-purified antibodies on HEp-2 cells;* **(A, B, C)** *show immunofluorescent patterns with affinity-purified antibodies from the 34 kDa band of serum W.K., from the 90 kDa doublet of serum I.I., and from the 37 kDa band of serum S.K., respectively, magnification ×800.*

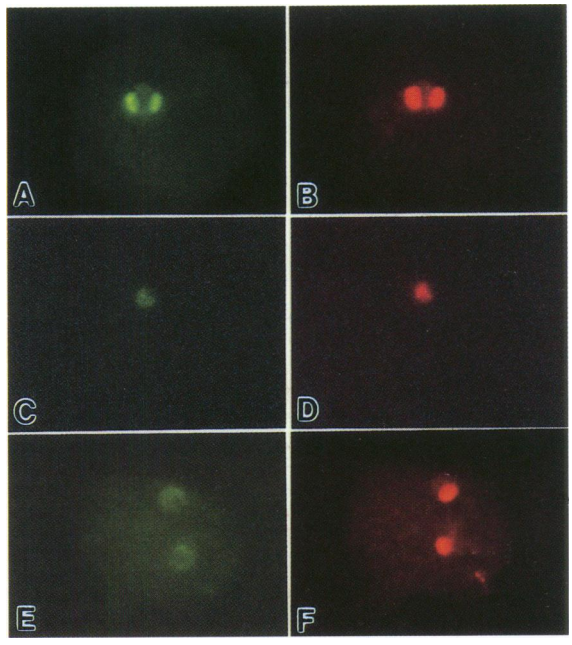

Figure 5. *PtK2 cells were treated with actinomycin D to segregate nucleoli into distinct fibrillar and granular regions. Double-label immunofluorescence was then used to show colocalization of antibodies. Serum W.K. (A-fluorescein) and monoclonal antibody to fibrillarin (B-rhodamine) localized in the fibrillar caps of the nucleolus. Serum S.K. (C-fluorescein) and monoclonal antibody to B23 (D-rhodamine) localized to the granular region of the nucleolus. Serum S.K. with antibody to B23 (E-fluorescein) and monoclonal antibody to fibrillarin (F-rhodamine) show staining of regions which are juxtaposed in the segregated nucleolus, magnification ×800.*

noelectron microscopy and immunofluorescence to be present in the fibrillar region and the 37 kDa protein B23 to be present in the granular region of such segregated nucleoli.[26] Two murine monoclonal antibodies, 72B9 reactive with fibrillarin and a murine monoclonal antibody reactive with protein B23, were available and could therefore be used in double-label immunofluorescence to determine if there was colocalization between the human cancer autoantibodies and the murine monoclonal antibodies of defined specificity. Figure 5A and 5B show the colocalization of serum W.K. and monoclonal antibody 72B9 in the fibrillar caps of the actinomycin D-segregated nucleolus, and Figure 5C and 5D show the colocalization of antibody from serum S.K. and murine monoclonal antibody to B23 in the granular region. Figure 5E and 5F show the complementary staining of an actinomycin D-segregated nucleolus with serum S.K. (human antibody reacting with the granular region) and 72B9 (monoclonal anti-fibrillarin reacting with the fibrillar region).

Further confirmation that the antigen recognized by S.K., W.S., and T.H. serum was protein B23 was demonstrated by two-dimensional immunoblotting (Figure 6). Both human sera and anti-B23 monoclonal antibody recognized a 37 kDa protein spot migrating at an identical isoelectric point of 5.0–5.5.

NORs are the regions on chromosomes that contain ribosomal RNA genes and they are present in different numbers in different species. There are five pairs of NORs in normal human diploid cells located on chromosomes 13, 14, 15, 21, and 22 and two or three pairs in Indian muntjac diploid cells, depending on the cell line.[39] Therefore, unlike centromeres which are present on all chromosomes, NORs are present only on selective chromosomes. Figure 7 shows immunofluorescence of NORs with serum from patient I.I. on spreads of Indian muntjac chromosomes (Figure 7A). Only two pairs of staining dots were observed in the diploid cell containing 7 chromosomes (cell on the left in 7A and 7B). NORs are also known to have affinity for silver staining[40] and this study demonstrates colocalization of immunofluorescence (Figure 7A) with silver staining (Figure 7B).

Clinical data and autoantibody profiles in the five patients are summarized in Table 2. Each of the three patients with HCC demonstrated reactivity with a different nucleolar protein. They were all positive for hepatitis B surface antigen (HBsAg) and were considered to be in advanced stages of liver cancer when the diagnosis was made. Findings suggestive of systemic autoimmune disease were not found during autopsy in these three patients. Pathologic findings revealed liver tumors that were widespread and associated with liver cirrhosis and multiple lymph-node metastases. The patients with lung adenocarcinoma and dysgerminoma were HBsAg negative. Metastasis to bone was present in both patients. As described in Materials and Methods, sera from these patients were obtained at the time when the diagnosis of cancer was made and before anti-cancer therapy was instituted.

Observations showing changing ANA titers are depicted in Figure 8 for four patients with HCC in whom multiple serum samples were available. In patients NK, Yo and Ta, ANA titers increased before HCC was detected and in patient Om, an increasing ANA titer was detected coincident with the diagnosis of HCC. In patient

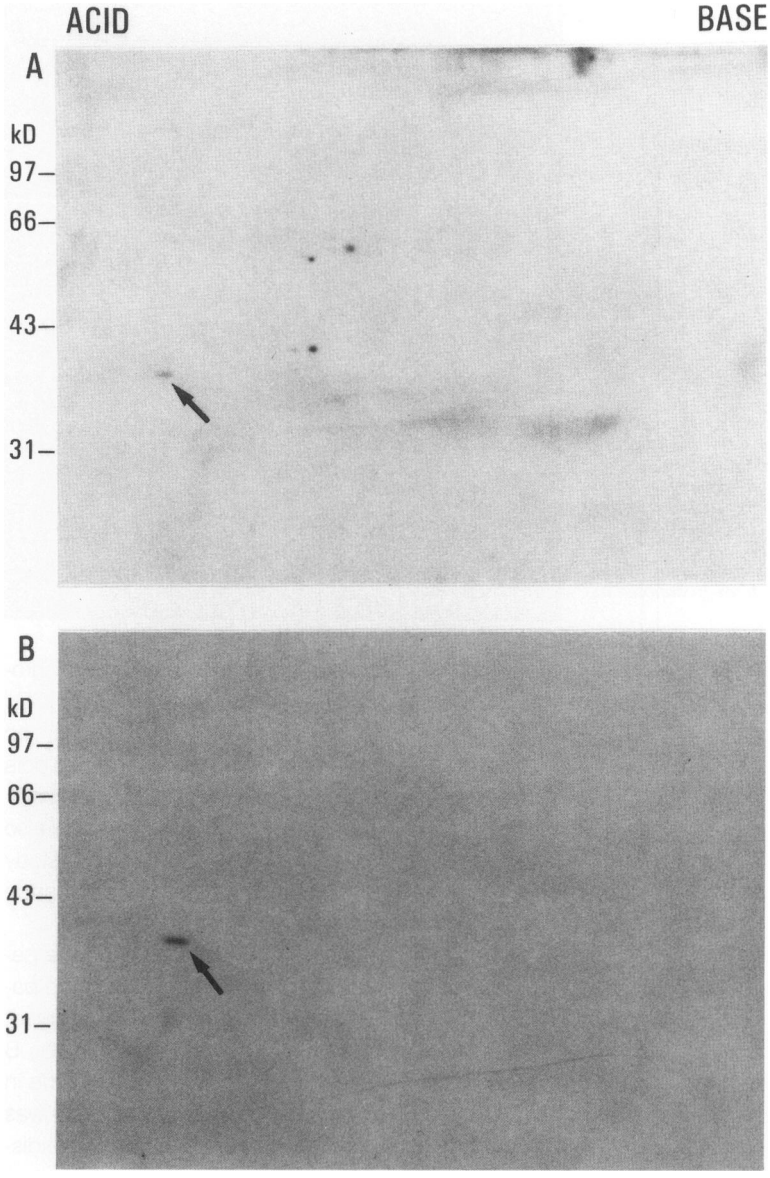

Figure 6. *Two-dimensional Western blotting analysis with HEp-2 cell nuclear extract, using serum from patient S.K. (upper panel) compared with monoclonal antibody to B23 (lower panel). Blotting of protein spots with an identical isoelectric point and molecular weight was demonstrated (arrows).*

NK, who had hepatitis B-related cirrhosis, the serum collected 16 months before diagnosis of HCC showed weak nucleoplasmic staining whereas strong nucleolar fluorescence was detected with the serum obtained at the time HCC was diagnosed (Figure 9). The other patients were ANA-negative and converted to ANA-positive status. Only in patient Om was an increase in serum α-fetoprotein detected, whereas no increase above normal was detected in the other three patients.

Discussion

The three antigens characterized in this study are nucleolar proteins that perform basic functions in normal cells and might be induced to greater activity during cell growth and proliferation. Fibrillarin is a component of the U3 RNP particle and is involved in the 5′ processing of precursor ribosomal RNA transcripts.[22] We have recently shown that the doublet proteins of NOR-90 are identical to hUBF (human upstream binding factor)[41] which activates ribosomal RNA transcription mediated by RNA polymerase I.[42] cDNAs encoding the protein doublet of NOR-90/hUBF have been cloned, the larger protein by Jantzen et al[42] and the smaller protein in this laboratory.[41] The coding regions of both these cDNAs are identical except for an internal deletion of 111 nucleotides in the smaller protein resulting in a polypeptide smaller by 37 amino acid residues. Nucleophosmin/protein B23 is lo-

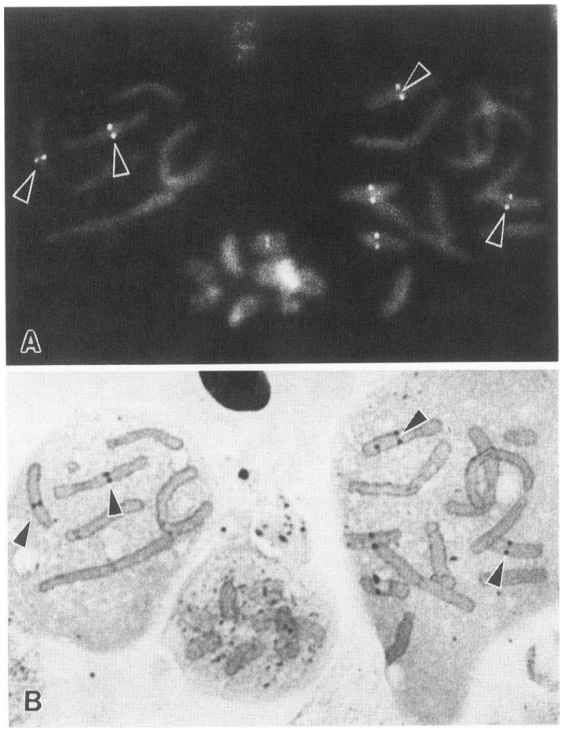

Figure 7. *Chromosomal spreads from Indian muntjac cells were reacted in immunofluorescence with serum of patient I.I. and then the same area was reacted with NOR silver staining reagents;* **(A)** *shows immunofluorescent staining of NORs on specific chromosomes (X and Y_1—arrowheads). The cell in the left half of the picture is diploid with 7 (2n) chromosomes, whereas the cell on the right is polyploid;* **(B)** *shows staining of the same NORs with silver (arrowheads), magnification ×800.*

calized to the granular regions of the nucleolus[43] and increased synthesis of this protein has been observed in cells committed to DNA replication and cell proliferation after mitogen activation.[23] These protein components of the nucleolus are all engaged in some aspect of ribosome biosynthesis, a process that might be increased in unregulated cell growth.

In the Western blotting study depicted in Figure 2A, it was shown that there was great diversity in the antigen-antibody systems. This diversity of autoantibodies in cancer has been confirmed in an expanded study that included 27 HCC sera examined by Western blotting (manuscript in preparation), and the question arises regarding a possible basis for this phenomenon.

The most common precursor conditions leading to HCC in this group of Japanese patients are liver cirrhosis and chronic hepatitis after infection with hepatitis B virus (HBV) or non-A, non-B hepatitis.[44] The biology of HBV has been extensively investigated and the relationship of HBV infection and HCC is well established.[45,46] Several pathways leading to carcinogenesis have been considered but at least two pathways that could be associated with production of autoantibodies to intranuclear antigens deserve mention. Insertional mutagenesis caused by integration of HBV DNA into cellular DNA has been considered to be a possible mechanism leading to cellular transformation.[45–49] HBV DNA integrations have been shown to occur at different sites in cellular DNA.[50] These integrations could result in dysregulation of different cellular genes, leading to alterations in a number of transcription and translation products. Also, the HBV X gene has been shown to have transcriptional transactivating properties and is capable of activating several different promoters, resulting in the overexpression of different gene products in transfected cells.[51,52] These effects of HBV might induce the abnormal expression of diverse intranuclear proteins that could stimulate autoimmune reactions. The possibility of intracellular antigens driving the autoimmune response has been considered in autoimmune disease.[19] Since some of the nucleolar antigens in HCC have also been detected in scleroderma, lupus, and Sjögren's syndrome[24,36,37,53,54] and are not unique to HCC, common mechanistic pathways might be the basis of the immune response. At the present time, we have no understanding of how the autoimmune response is initiated. This is clearly an important aspect of future studies.

Some patients developing HCC from pre-existing chronic liver disease sero-converted from ANA-negative to ANA-positive status or showed changes in ANA specificity in close temporal relationship with clinical changes. This raises the question whether changes in ANA responses might reflect early carcinogenesis at time points when cancer nodules were small enough that they es-

Table 2. *Clinical Data and Antibody Specificities in Five Cancer Patients with Antinucleolar Antibodies*

				Antibodies to cellular antigens, Western blotting—Mr (kDa)	
Patient	Sex	Age	Diagnosis	Identified	Unidentified
WK	M	52	HCC*	34 (fibrillarin)	56, 80, 99
I I	M	52	HCC	93/89 (NOR-90)	
WS	M	45	HCC	37 (B23)	57
SK	M	70	Lung adenocarcinoma	37 (B23)	41
TH	F	65	Dysgerminoma	37 (B23)	

* Hepatocellular carcinoma.

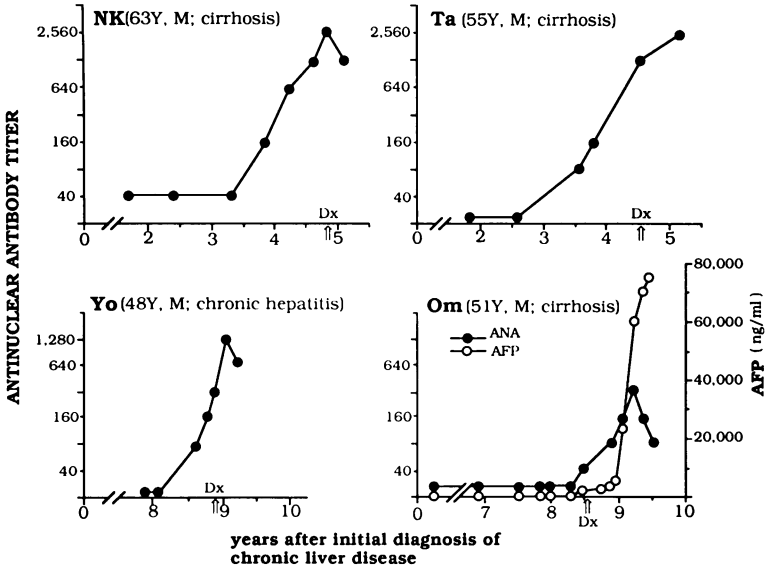

Figure 8. *Longitudinal studies in four patients who developed HCC from cirrhosis or chronic hepatitis. Reciprocal titer of ANAs was determined by indirect immunofluorescence on HEp-2 cells using anti-human IgG FITC-conjugate as second antibody. Dx indicates time point when diagnosis of HCC was made. Note that increasing titer of ANAs preceded diagnosis of HCC in patients NK, Ta, and Yo.*

caped detection at routine clinical examinations. The current observations were made in a small number of patients because it was a retrospective study and only a limited number of serial serum samples were available. Nevertheless, the data point to the dynamic nature of autoimmune responses in the transition to HCC and give further credibility to the possibility that autoantigens might be driving the immune reaction. This issue should be examined in prospective studies of larger patient populations with liver cirrhosis and chronic hepatitis, conditions that have a high risk of progression to HCC. Further studies are needed to establish the antigenic specificities of other ANAs in HCC. This information could be helpful in formulating a better understanding of the basis of autoantibody responses in malignant conditions. It will be important to establish whether autoantibodies in HCC and other malignancies are somewhat selective and directed against specific intracellular proteins that might be preferentially activated in cell functions related to growth and proliferation.

The observations in this study raise important considerations regarding *in vivo* mechanisms that stimulate immune responses to intracellular antigens. The fact that different diseases like scleroderma and HCC result in the formation of autoantibody to NOR-90 and fibrillarin, and Sjögren's syndrome and three types of cancer including HCC result in autoantibody to protein B23 demonstrate that these autoantibody responses are not specific or uniquely related to a disease entity. There should however be a common factor or factors driving the immune response to cause autoantibody formation to the same intracellular antigen(s) and elucidation of this feature might contribute to further understanding of autoimmune reactions.

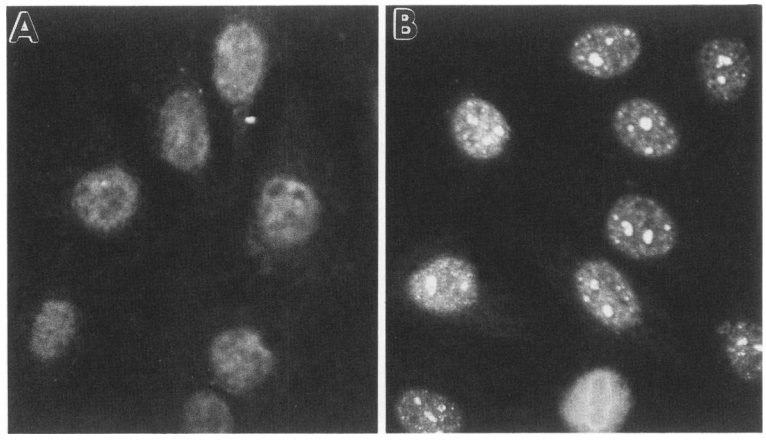

Figure 9. *Changing autoantibody specificity as observed by immunofluorescence on HEp-2 cells in patient NK;* **(A)** *depicts immunofluorescence with serum obtained 16 months before HCC was detected;* **(B)** *demonstrates immunofluorescence with serum collected at diagnosis of HCC, magnification x800.*

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References

1. Whitehouse JMA, Holborow EJ: Smooth muscle antibody in malignant disease. Br Med J 1971, 27:511–513
2. Burnham TK: Antinuclear antibodies in patients with malignancies. Lancet 1972, 2:436–437
3. Steiner M, Klein E, Klein G: Antinuclear reactivity of sera in patients with leukemia and other neoplastic diseases. Clin Immunol Immunopathol 1975, 4:374–381
4. Thomas PJ, Kaur JS, Aitcheson CT, Robinson WA, Tan EM: Antinuclear, antinucleolar, and anticytoplasmic antibodies in patients with malignant melanoma. Cancer Res 1983, 43:1372–1380
5. Hodson ME, Turner-Warwick M: Autoantibodies in patients with bronchial carcinoma. Thorax 1975, 30:367–370
6. Wasserman J, Glas U, Blomgren H: Autoantibodies in patients with carcinoma of the breast: Correlation with prognosis. Clin Exp Immunol 1975, 19:417–422
7. Schattner A, Shani A, Talpaz M, Bentwich Z: Rheumatoid factors in the sera of patients with gastrointestinal carcinoma. Cancer 1983, 52:2156–2161
8. Nelson DS: Autoantibodies in cancer patients. Pathology 1977, 9:155–158
9. Lamelin JP, de-The G, Revillard JP, Gabbiani G: Autoantibodies (cold lymphocytotoxins, antiactin antibodies and antinuclear factors) in nasopharyngeal carcinoma patients. IARC Sci Publ 1978, 20:523–536
10. Ablin RJ: Malignancy associated with antinuclear antibodies. Lancet 1972, 2:1253–1254
11. Kiyosawa K, Imai H, Sodeyama T, Furuta K, Gibo Y, Kumagai T, Kameko M, Kanai M, Furuta S: Serum autoantibodies in patients with hepatocellular carcinoma: The clinical significance of antinuclear antibody. J Gastroenterol Hepatol 1986, 1:371–378
12. Budde-Steffen C, Anderson NE, Rosenblum MK, Posner JB: Expression of an antigen in small cell lung carcinoma lines detected by antibodies from patients with paraneoplastic dorsal root ganglionopathy. Cancer Res 1988, 48:430–434
13. Bonfa E, Bystryn J-C, Elkon KB: Detection of immunoglobulin G antibodies in melanoma sera reactive with intracellular proteins. J Invest Dermatol 1988, 90:207–212
14. Chen Y-M, Hu C-P, Chen P-H, Chu M-HH, Tsai Y-T, Lee S-D, Chang C: Nuclear antigens reacted with sera and ascites of hepatocellular carcinoma patients. Hepatology 1988, 8:547–552
15. Kiyosawa K, Daemer RJ, He L-F, Bonino F, Prozesky OW, Purcell RH: The spectrum of complement-fixing antinuclear antibodies in patients with hepatocellular carcinoma. Hepatology 1985, 5:548–555
16. Polans AS, Buczylko J, Crabb J, Palczewski K: A photoreceptor calcium binding protein is recognized by autoantibodies obtained from patients with cancer-associated retinopathy. J Cell Biol 1991, 112:981–989
17. Sakai K, Mitchell DJ, Tsukamoto T, Steinman L: Isolation of a complementary DNA clone encoding an autoantigen recognized by an anti-neuronal cell antibody from a patient with paraneoplastic cerebellar degeneration. Ann Neurol 1990, 28:692–698
18. Fathallah-Shaykh H, Wolf S, Wong E, Posner JB, Furneaux HM: Cloning of a leucine-zipper protein recognized by the sera of patients with antibody-associated paraneoplastic cerebellar degeneration. Proc Natl Acad Sci USA 1991, 88:3451–3454
19. Tan EM: Antinuclear antibodies: Diagnostic markers for autoimmune diseases and probes for cell biology. Adv Immunol 1989, 44:93–151
20. Prestayko AW, Klomp GR, Schmoll DJ, Busch H: Comparison of proteins of ribosomal subunits and nucleolar preribosomal particles from Novikoff hepatoma ascites cells by two-dimensional polyacrylamide gel electrophoresis. Biochemistry 1974, 13:1945–1951
21. Yung BY-M, Busch H, Chan P-K: Translocation of nucleolar phosphoprotein B23 (37kDa/pI 5.1) induced by selective inhibitors of ribosome synthesis. Biochim Biophys Acta 1985, 826:167–173
22. Kass S, Tyc K, Steitz JA, Sollner-Webb B: The U3 small nucleolar ribonucleoprotein functions in the first step of preribosomal RNA processing. Cell 1990, 60:897–908
23. Feuerstein N, Chan PK, Mond JJ: Identification of numatrin, the nuclear matrix protein associated with induction of mitogenesis, as the nucleolar protein B23: Implication for the role of the nucleolus in early transduction of mitogenic signals. J Biol Chem 1988, 263:10608–10612
24. Rodriguez-Sanchez JL, Gelpi C, Juarez C, Hardin JA: Anti-NOR90: A new autoantibody in scleroderma that recognizes a 90-kDa component of the nucleolus-organizing region of chromatin. J Immunol 1987, 139:2579–2584
25. Lischwe MA, Ochs RL, Reddy R, Cook RG, Yeoman LC, Tan EM, Reichlin M, Busch H: Purification and partial characterization of a nucleolar scleroderma antigen (Mr = 34,000; pI, 8.5) rich in N^G, N^G-dimethylarginine. J Biol Chem 1985, 15:14304–14310
26. Ochs RL, Lischwe MA, Spohn WH, Busch H: Fibrillarin: A new protein of the nucleolus identified by autoimmune sera. Biol Cell 1985, 54:123–134
27. Reimer G, Pollard KM, Penning CA, Ochs RL, Lischwe MA, Busch H, Tan EM: Monoclonal antibody from a (New Zealand black × New Zealand white) F1 mouse and some human scleroderma sera target an Mr 34,000 nucleolar protein of the U3 RNP particle. Arthrit Rheum 1987, 30:793–800
28. Ochs R, Lischwe M, O'Leary P, Busch H: Localization of nucleolar phosphoproteins B23 and C23 during mitosis. Exp Cell Res 1983, 146:139–149
29. Merry DE, Pathak S, Hsu TC, Brinkley BR: Anti-kinetochore antibodies: Use as probes for inactive centromeres. Am J Hum Genet 1985, 37:425–430

30. Ploton D, Menager M, Jeannesson P, Himber G, Pigeon F, Adnet JJ: Improvement in the staining and in the visualization of the argyrophilic proteins of the nucleolar organizer region at the optical level. Histochem J 1986, 18:5–14
31. Muramatsu M, Hayashi Y, Onishi T, Sakai M, Takai K, Kashiyama T: Rapid isolation of nucleoli from detergent purified nuclei of various tumor and tissue culture cells. Exp Cell Res 1974, 88:345–351
32. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970, 227:680–685
33. O'Farrell PZ, Goodman HM, O'Farrell PH: High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell 1977, 12:1133–1142
34. Burke B, Griffiths G, Reggio H, Louvard D, Warren G: Monoclonal antibody against a 135-K golgi membrane protein. EMBO J 1982, 1:1621–1628
35. Tan EM: Autoantibodies to nuclear antigens (ANA): Their immunology and medicine. Adv Immunol 1982, 33:167–240
36. Pfeifle J, Anderer FA, Franke M: Characterisation of nucleolar proteins as autoantigens using human autoimmune sera. Ann Rheum Dis 1986, 45:978–986
37. Reimer G, Steen VD, Penning CA, Medsger TA, Jr, Tan EM: Correlates between autoantibodies to nucleolar antigens and clinical features in patients with systemic sclerosis (scleroderma). Arthrit Rheum 1988, 31:525–532
38. Phillips SG, Phillips DM: Nucleoli of diploid cell strains: Their normal ultrastructure and the effects of toyocamycin and actinomycin D. J Cell Biol 1971, 49:785–802
39. Howell WM: Selective staining of nucleolus organizer regions (NORs). The Cell Nucleus, rDNA, Part B. Vol XI. Edited by Busch H, Rothblum LI. New York, Academic Press, 1982, pp 89–142
40. Goodpasture C, Bloom SE: Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. Chromosoma 1975, 53:37–50
41. Chan EKL, Imai H, Hamel JC, Tan EM: Human autoantibody to RNA polymerase I transcription factor hUBF: Molecular identity of nucleolus organizer region autoantigen NOR-90 and ribosomal RNA transcription upstream binding factor. J Exp Med 1991, 174:1239–1244
42. Jantzen H-M, Admon A, Bell SP, Tjian R: Nucleolar transcription factor hUBF contains a DNA-binding motif with homology to HMG proteins. Nature 1990, 344:830–836
43. Spector DL, Ochs RL, Busch H: Silver staining, immunofluorescence, and immunoelectron microscopic localization of nucleolar phosphoproteins B23 and C23. Chromosoma 1984, 90:139–148
44. Okuda K, Fujimoto I, Hanai A, Urano Y: Changing incidence of hepatocellular carcinoma in Japan. Cancer Res 1987, 47:4967–4972
45. Popper H, Shafritz DA, Hoofnagle JH: Relation of the hepatitis B virus carrier state to hepatocellular carcinoma. Hepatology 1987, 7:764–772
46. Tabor E: Strongly supported features of the association between hepatitis B virus and hepatocellular carcinoma. Etiology, Pathology, and Treatment of Hepatocellular Carcinoma in North America. Edited by Tabor E, DiBisceglie AM, Purcell RH. London, Gulf Publishing Co., 1991, pp 107–117
47. Matsubara K, Tokino T: Integration of hepatitis B virus DNA and its implications for hepatocarcinogenesis. Mol Biol Med 1990, 7:243–260
48. Kekulé AS, Lauer U, Meyer M, Caselmann WH, Hans Hofschneider P, Koshy R: The preS2/S region of integrated hepatitis B virus DNA encodes a transcriptional transactivator. Nature 1990, 343:457–461
49. Slagle BL, Zhou Y-Z, Butel JS: Hepatitis B virus integration event in human chromosome 17p near the p53 gene identifies the region of the chromosome commonly deleted in virus-positive hepatocellular carcinomas. Cancer Res 1991, 51:49–54
50. Tiollais P, Pourcel C, Dejean A: The hepatitis B virus. Nature 1985, 317:489–495
51. Spandau DF, Lee C-H: trans-activation of viral enhancers by the hepatitis B virus X protein. J Virol 1988, 62:427–434
52. Koshy R, Zahm P, Wollersheim M, Hofschneider PH: Transactivation by HBV X gene product. Liver Cell Carcinoma. Edited by Bannasch P, Keppler D, Weber G. London, Kluwer Academic, 1989, pp 117–133
53. Li X, McNeilage LJ, Whittingham S: Autoantibodies to the major nucleolar phosphoprotein B23 define a novel subset of patients with anticardiolipin antibodies. Arthrit Rheum 1989, 32:1165–1169
54. Kindas-Mügge I: Human autoantibodies against a nucleolar protein. Biochem Biophys Res Commun 1989, 163:1119–1127