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Immunohistochemical and Semiquantitative Immunoblot Analyses of Nm23-H1 and H2 Isoforms in Normal Human Tissues

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The total amount of nm23 protein, the relative ratios of H1 and H2 isoforms (H2/H1) and the localization of these proteins in human normal tissues were studied by a semiquantitative immunoblot technique followed by densitometry and immunohistochemistry with monoclonal antibody against nm23 protein (Pan-242). All tissues contained both isoforms recognized as the 20.5 kD H1 protein and 18.5 kD H2 protein by immunoblotting. Nm23 protein was abundant in liver, kidney and adrenal gland tissue, and scarce in heart and muscle. H2 levels were always higher than H1, but the isoform ratios (H2/H1) were variable from tissue to tissue. Immunostaining revealed that nm23 protein was predominantly present in cytoplasm and the pattern of staining was homogeneous in parenchymal cells of the liver, pancreas and colonic mucosa and heterogeneous in gastric mucosa and kidney. These results demonstrated that the levels of nm23 protein and the H2/H1 ratios and distribution of isoforms were different in each tissue, and suggests that, when the alterations of nm23 gene expression in tumor tissues are examined, the levels and ratios in non-tumorous tissues surrounding the tumor nest should be considered.

Key words: nm23-H1, nm23-H2, Immunoblotting, Immunohistochemistry, AMeX-fixed tissue

I. Introduction

The nm23 gene was first shown to be a novel metastasis-suppressor gene by differential colony hybridization between two murine melanoma sublines, one with high and the other with low metastatic potential [28]. Subsequently, Leone *et al.* [18] demonstrated that transfection of nm23 cDNA into a highly metastatic K-1735 subline showed significantly reduced metastatic potential. These findings suggested that the nm23 gene may be directly implicated in the mechanism of cancer metastasis. Two isoforms were isolated from human nm23 protein and named nm23-H1 and nm23-H2 [23, 28].

The alteration of nm23 gene expression in various types of human cancer has been examined as a potential prognostic factor. An inverse correlation between the level of nm23 mRNA or protein and metastatic potential was reported in breast cancers [5, 9, 11]. Similar results were also obtained in hepatocellular carcinomas, gastric carcinomas and malignant melanomas [6, 20, 21]. However, there was a positive correlation between nm23 expression and metastatic potential in advanced stage colon carcinomas [8], neuroblastomas [7] and pancreatic carcinomas [19], and no correlation was found in adenocarcinoma of the lung and endometrial carcinoma [10, 34]. Similarly, we found different correlations between nm23 gene expression and metastatic potential in different tumors [15, 34]. Recent studies have revealed different levels of nm23-H1 and H2 protein expression in some cancers [16, 30]. The expression of each isoform in normal rat tissues has been studied [27], but not in humans.

In this study, we examined the levels of nm23 protein, both isoforms and the relative ratios of H1 and H2 isoforms in normal human tissues by a semiquantitative immunoblotting method using a chemiluminescence detection system and densitometry, and investigated the precise

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location of proteins with AMeX-fixed and paraffin-embedded tissue sections [25] using immunohistochemistry. AMeX fixation of tissue preserves many antigens as well as high-molecular weight DNA, RNA and protein, which is destroyed by the formalin fixation and paraffin-embedding process [26].

The results demonstrated that the levels of nm23 protein and the relative ratios of both isoforms (H2/H1), and the distribution of isoforms were different in different tissues.

II. Materials and Methods

Preparation of monoclonal antibody

The entire coding regions of nm23-H1, nm23-H2, nm23-M1 and nm23-M2 gene were translated as fusion proteins with 26-kD glutathione S-transferase (GST) in E. coli. A rat was immunized three times with nm23-M2 fusion protein at two week intervals: the first time subcutaneously with 50 μg of protein and complete Freund's adjuvant, the second time subcutaneously with 100 μg of protein and incomplete Freund's adjuvant, and the third time intravenously with 100 μg of fusion protein alone. Three days after the final immunization, spleen cells were obtained from the immunized rat and fused with murine myeloma NS-1 cells. The hybridoma culture supernatants were assayed for reactivity with the nm23-H1 and nm23-H2 proteins using an enzyme-linked immunosorbent assay and immunoblotting [32, 33]. Limiting dilution of positive clones was performed three times to obtain monoclonals. The monoclonal antibody (mAb) Pan-242, specific for all of the human and murine nm23 proteins was obtained. The specificity of Pan-242 antibody was confirmed by immunoblotting using the lysate from nm-23-H1 or -H2 fusion proteins [33].

Tissues

Tissues were obtained from patients with several different cancers at surgery or autopsy within 4 hr of postmortem at Kitasato University Hospital. They did not exhibit any tumor cell invasion or any significant inflammatory changes. In total, 25 lungs, 9 thyroid glands, 8 livers, 7 colonic mucosa, 7 gastric mucosae, 6 kidneys, 5 pancreases, 5 adrenal glands, 5 mammary glands, 5 hearts, 5 spinal cords, 4 skeletal muscles, 3 brains (gray matter and white matter were separated) and 3 salivary glands were obtained. Each tissue was divided into two parts. One part was snap frozen and stored at $-80°C$ until just before use, and the remainder was fixed by the AMeX method [25] and stored at 4°C.

Immunoblotting

Briefly, about 50 mg wet-weight of each of the frozen tissues was minced and homogenized in the extraction buffer [62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% Glycerol, 5% 2-mercaptoethanol (2-ME), 0.015% phenyl methyl sulfonyl fluoride (PMSF), 0.001% bromophenol blue (BPB)] as described previously [17]. After centrifugation at 15000 r.p.m. for 10 min at 4°C, the supernatant was collected and protein concentration was measured using a Bio-Rad Protein Assay kit (Bio-Rad Lab., Hercules, CA, USA) and adjusted to 2 mg/ml with extraction buffer. The resulting lysate (20 μg protein/lane) was loaded onto a 15% SDS-polyacrylamide gel. Simultaneously, 0.3 μg of each protein extracted from murine myeloma cell lines, NS-H1-9 and NS-H2-1, which were transfected with the nm23-H1 and -H2 genes, respectively [31], was loaded as positive controls. After electrophoresis, the protein was transferred to a polyvinylidene difluoride membrane (Immobilon; Japan Millipore Limited, Tokyo). Subsequently, the membrane was preincubated with Block Ace (Dainippon Seiyaku, Osaka) for 2 hr at room temperature to block nonspecific binding of protein. The membrane was then incubated with Pan242 antibody (1 : 10) for 1 hr at room temperature. After rinsing in 0.05% (V/V) Tween20-phosphate-buffered saline (T-PBS) five times for 5 min each, the membrane was incubated with 1000-fold diluted peroxidase conjugated anti-rat IgG (DAKO JAPAN, Kyoto) for 30 min at room temperature. After rinsing in T-PBS as mentioned above, the membrane was reacted with Western Blot Chemiluminescence Reagent (Renaissance: DuPont, Boston, MA, USA.) according to the manufacturer's instructions. Finally, the membrane was exposed to FUJI RX Film (FUJI PHOTO FILM, Tokyo), and the signal exposures were developed.

Quantitative analysis of protein levels with densitometry

To obtain the range in which the signals detected by the immunoblotting were proportional to the amount of protein loaded, a preliminary study was performed. The protein lysate extracted from NS-H2-1 cells was diluted with the extraction buffer [17] in eight grades from 640 ng to 40 ng and subjected to immunoblotting as described above. The densities of each signal were measured with a densitometer (CS9000: SHIMADZU, Kyoto) at 550 nm. The level of H1 and H2 isoforms in each case was presented as a relative amount compared with the level of 0.3 μg lysate protein of NS-H2-1 cells loaded on the same gel.

Immunostaining

Three micrometer thick sections from AMeX-fixed and paraffin-embedded tissues were stained immunohistochemically by the avidin-biotin-peroxidase complex (ABC) method. In brief, the sections were deparaffinized in xylene and acetone, then refixed in 4% paraformaldehyde (PFA) before rehydration. Subsequently, the sections were rinsed in PBS and incubated with 2% normal swine serum in PBS for 10 min at room temperature to block nonspecific antibody binding. The sections were incubated with 10-fold diluted Pan-242 antibody overnight at room temperature. After rinsing three times in PBS for 5 min each, the sections were incubated with 200-fold

diluted biotinylated goat anti-rat immunoglobulin (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. After rinsing in PBS, the sections were treated with methanol containing 0.3% hydrogen peroxide (H_2O_2) for 20 min at room temperature to suppress endogenous peroxidase activity. Then the sections were incubated with 100-fold diluted streptavidin-biotin-peroxidase complex (Amersham Int., Buckinghamshire, UK) for 30 min at room temperature. Finally, reaction products were visualized by 0.02% 3,3′-diaminobenzidine tetrahydrochloride and 0.03% H_2O_2 in 0.1 M Tris-HCl buffer (pH 7.4). Nuclear counterstaining was performed with Mayer's hematoxylin solution. Immunostaining intensity was roughly evaluated as strongly positive, weakly positive (almost the same intensity) and negative (reduced intensity) in comparison with the staining intensity of fibroblasts within the same section.

Ten normal pancreatic tissues fixed with 10% formalin and embedded in paraffin were also stained in the same way as described above to compare the stainability of nm23 protein in AMeX-fixed and paraffin-embedded tissues.

III. Results

The specificity of Pan-242 antibody was confirmed by immunoblotting (Fig. 1). This antibody reacted with nm23-H1 or -H2 GST fusion protein at the same intensity, but not with GST protein alone. Densitometric analysis also confirmed that the signal densities between the two isoforms were equivalent (data not shown).

Next, we examined whether the levels of nm23 protein could be semiquantitatively analyzed by our immunoblotting method. The resulting lysates extracted from NS-H2-1 cells were diluted in eight grades and subjected to immunoblotting using Pan-242 antibody (Fig. 2). The signals were gradually decreased according to the dilution rate. Densitometric analysis showed that the signal densities were in proportion to the protein levels from 60 to 640 ng protein (5000 to 120000 as a densitometric value). As the densities of nm23 protein examined in this study were all within this range, we confirmed that the protein expression level could be analyzed semiquantitatively by this method.

Whole protein samples from human normal tissues were subjected to immunoblot analysis using Pan-242 antibody (Fig. 3). A band at 20.5 kD was only detected in NS-H1-9 cells, and a band at 18 kD was detected in NS-H2-1 cells, corresponding to the nm23-H1 and H2 isoforms, respectively. In NS-H1-9 cells, there was another band at 19 kD in addition to the major band. This band was not found in any human normal tissues. So, this might be a murine-derived protein from NS-1 myeloma cells. Almost all tissues contained both isoforms, but the levels varied from tissue to tissue. In muscle and heart, a clear band corresponding to nm23-H2 protein was detected, but nm23-H1 protein levels were very low. Fig. 4 shows the relative ratio of the two nm23 isoforms in each tissue. The relative amount 1.0 is equivalent to 0.3 μg of NS-H2-1 protein lysate as described in Materials and Methods. The liver contained the largest amount of nm23 protein, followed by the kidney and adrenal gland. Mammary gland, heart and muscle tissue contained low levels of both proteins. The levels of nm23-H1 protein in heart and muscle were out of the range of sensitivity of the densitometer. H2 isoform levels were higher than H1 protein in all tissues, but the H2/H1 ratios varied from 1.5 (thyroid) to 6.1 (lung). In the gray matter of the brain and thyroid, levels of both proteins were almost equal, but in the other tissues H2 protein levels were at least double those of H1 protein. In brain tissue, the total amount of nm23 protein was higher in the gray matter, but the H2/H1 ratio was higher in the white matter. These data demonstrated that the total amount of nm23 protein, the level of each isoform and H2/H1 ratios varied between tissues, and also varied in different regions within the same tissue such as the brain.

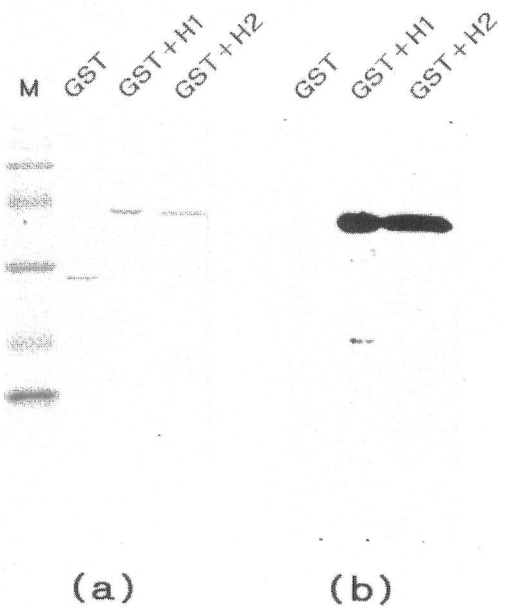

Fig. 1. Immunological characterization of Pan-242 mAb. **(a)** 500 μg each of protein lysate from nm23-H1 and H2 fusion proteins with 26 kD GST, and GST alone as described in Materials and Methods were fractionated by 15% SDS-PAGE and stained with Coomassie blue. Both fusion protein and GST alone showed a single band at 46 kD and 26 kD, respectively. Molecular size markers (M) are from bottom to top: lysozyme (14,400); trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), serum albumin (66,200), and phosphorylase b (97,400). **(b)** Immunoblotting with Pan-242 mAb of fusion proteins as described in **(a)**. Both nm23-H1 and H2 fusion proteins were stained with the same intensity at 46 kD, whereas GST alone was not stained with this antibody.

We examined the localization of nm23 protein by immunostaining on AMeX-fixed and paraffin-embedded tissues (Fig. 5). Nm23 protein showed a predominantly cytoplasmic staining pattern. Tissues were subdivided

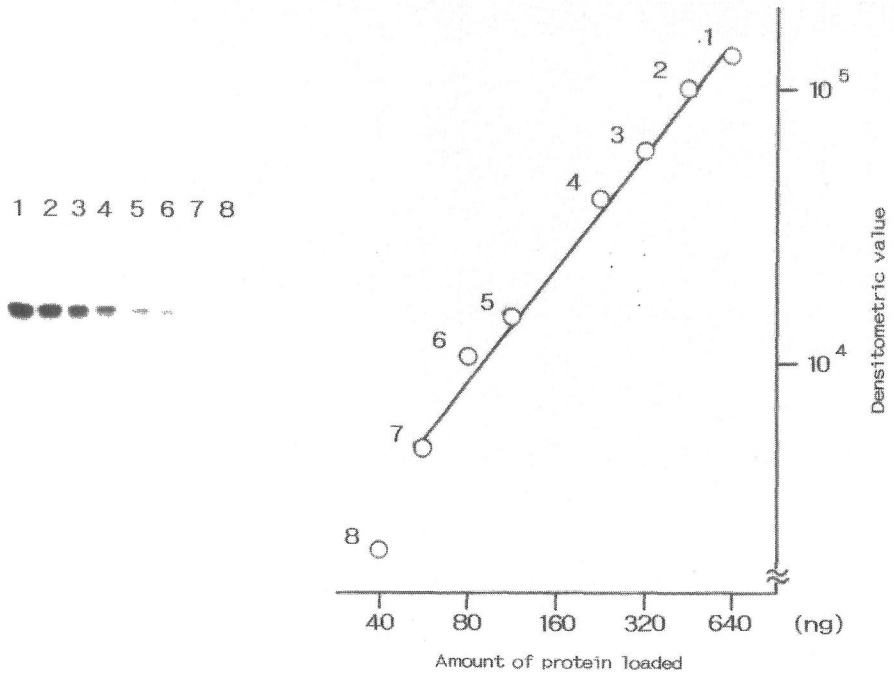

Fig. 2. The relationship between the concentration of nm23 protein in NS-H2-1 cells with Pan-242 antibody and densitometric values. Left: The signals gradually decreased according to the dilution rate. Right: The densitometric values of nm23 protein with Pan-242 antibody was in proportion to the concentration of protein within the range of 60 ng to 640 ng (densitometric values from 5000 to 120000).

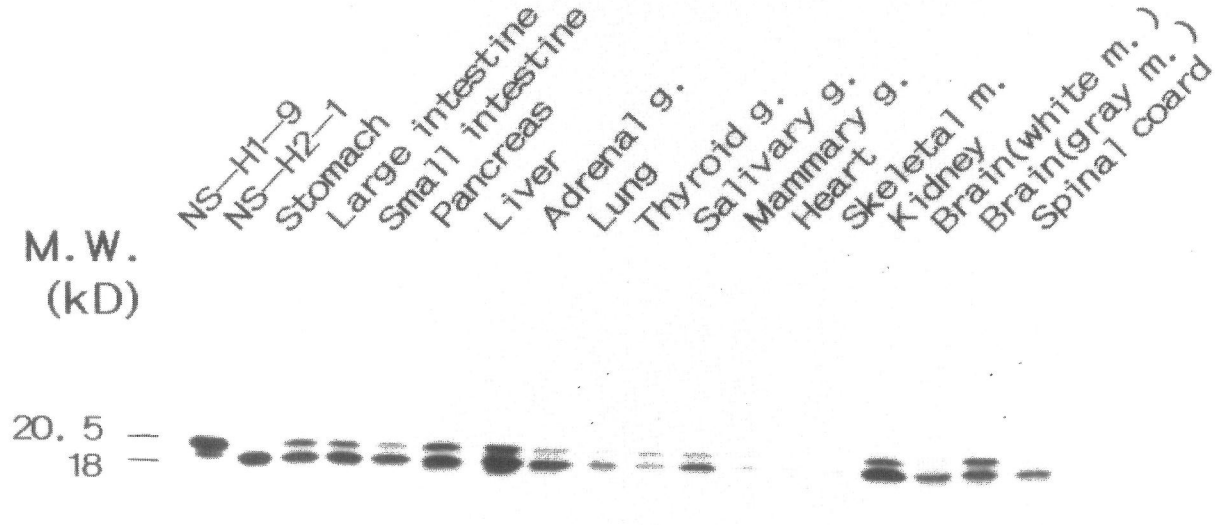

Fig. 3. Immunoblotting of extracts from NS-H1-9 cells, NS-H2-1 cells and human normal tissues incubated with Pan-242 antibody. A 20.5 kD band corresponding to nm23-H1 isoform and a weak band at 19 kD were detected in NS-H1-9 cells, and an 18 kD band corresponding to the nm23-H2 isoform was detected in NS-H2-1 cells. All tissues except for muscle and heart contained both isoforms but the levels varied from tissue to tissue. In muscle and heart, the nm23-H1 isoform was not discernible.

into two groups by the pattern of nm23 protein staining. Liver, in which the level of nm23 protein was the highest by immunoblotting, showed an intense homogeneous staining pattern throughout the hepatic parenchyma (Fig. 5A). Pancreatic parenchymal cells including islet cells, acinar cells, and duct epithelium were stained intensely (Fig. 5B). Intense staining was also observed throughout colon mucosa. Heart tissue was stained weakly at almost the same level as fibroblasts, and nm23 protein levels were also very low by immunoblotting (Fig. 5C). Heterogeneity in intensity was observed in adrenal gland, kidney, gray matter of brain, gastric mucosa and thyroid tissues. In the adrenal gland, the cortex showed stronger staining than that in the medulla (Fig. 5D). In the kidney, the

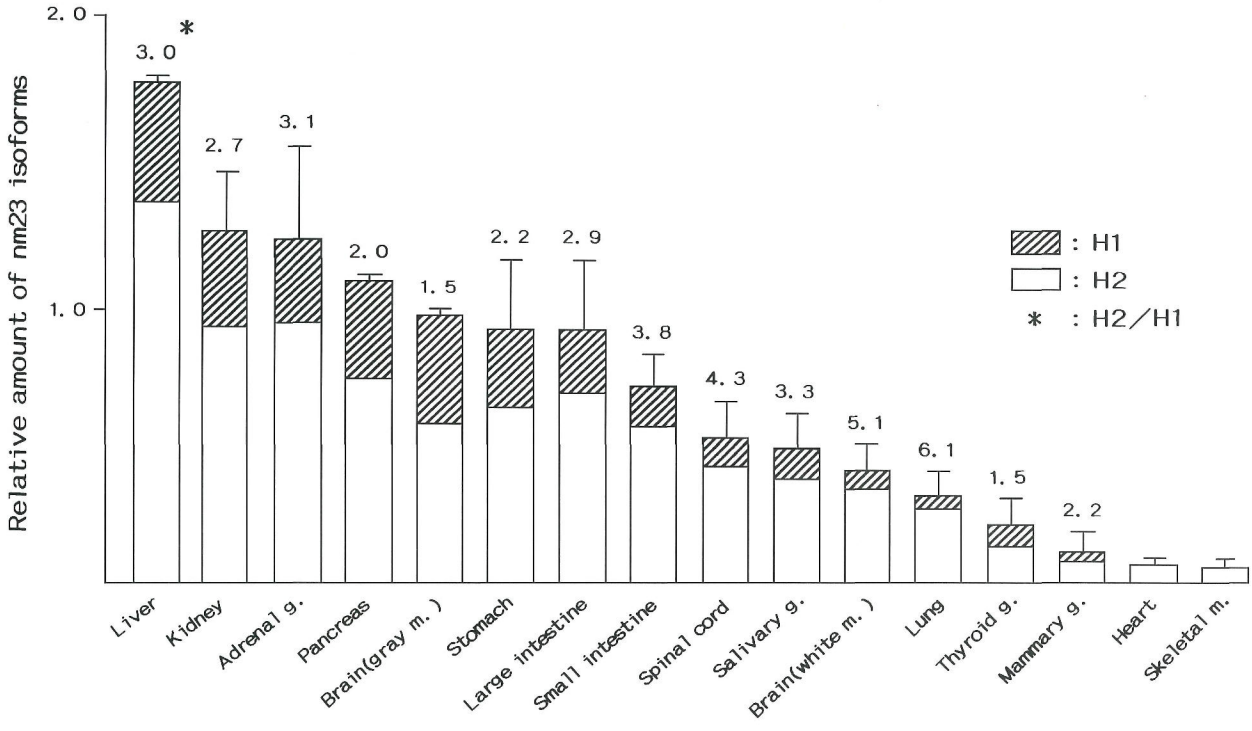
Relative amount of nm23 isoforms
2. 0
1. 0
*
3. 0
2. 7
3. 1
2. 0
1. 5
2. 2
2. 9
3. 8
4. 3
3. 3
5. 1
6. 1
1. 5
2. 2
: H1
: H2
* : H2/H1
Liver
Kidney
Adrenal g.
Pancreas
Brain(gray m.)
Stomach
Large intestine
Small intestine
Spinal cord
Salivary g.
Brain(white m.)
Lung
Thyroid g.
Mammary g.
Heart
Skeletal m.

Fig. 4

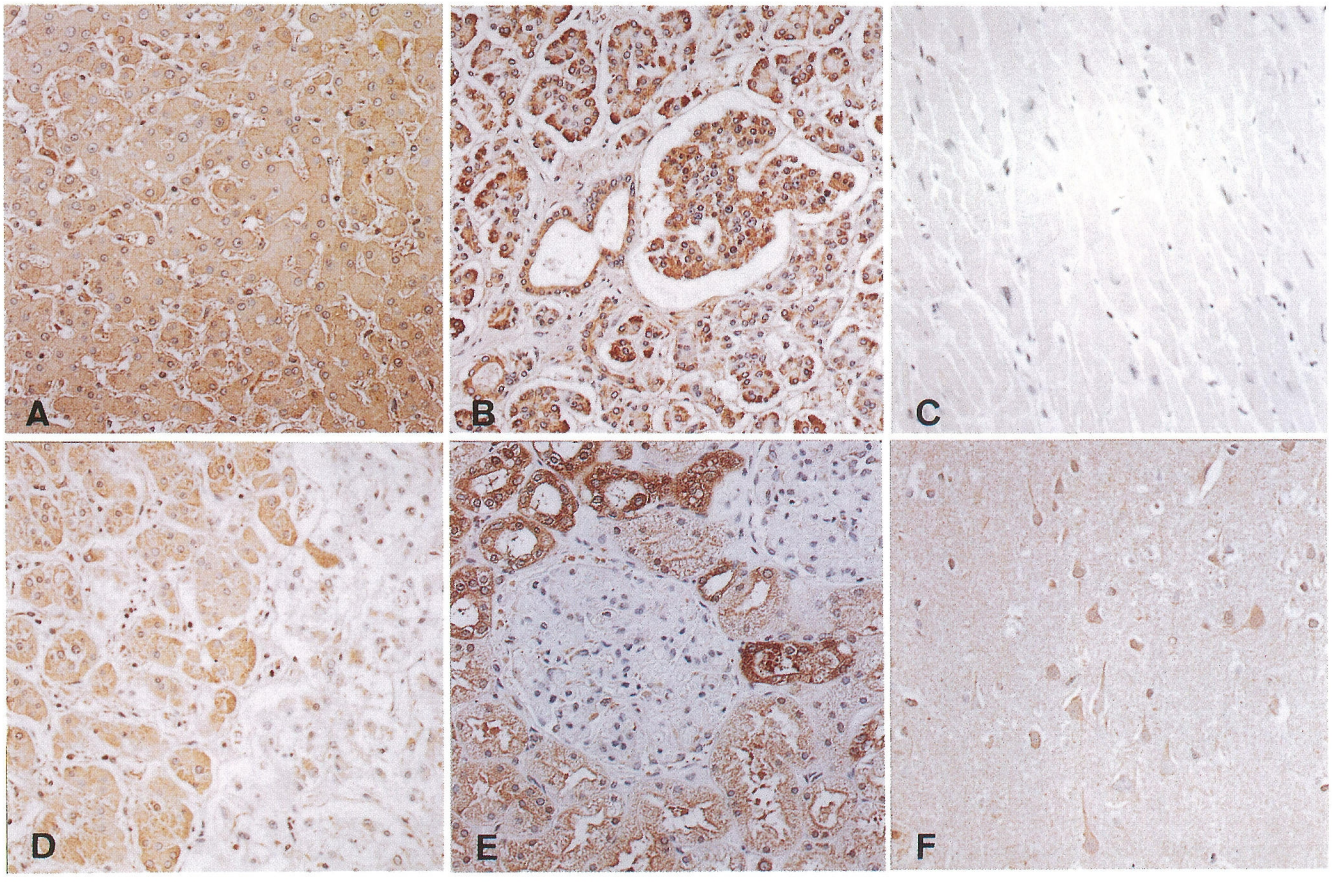
A
B
C
D
E
F

Fig. 5

glomerulus showed no immunoreactivity, but the renal tubule epithelium showed moderate to strong immunoreactivity. Distal tubules were more strongly stained than proximal ones (Fig. 5E). In gastric mucosa, the intensity was weak in foveola, moderate in neck and strong in basal epithelium. In the thyroid gland, some of the epithelial cells were stained weakly and others showed no reactivity. The remaining tissues showed homogeneous weak to moderate staining. In the homogeneously immunostained group, the tissues which were categorized as strongly positive in AMeX-fixed and paraffin-embedded tissue sections showed high nm23 protein levels by immunoblotting, and the tissues which were categorized as weakly positive showed low protein levels (Fig. 4). Generally, the stainability of nm23 protein in 10% formalin-fixed and paraffin-embedded tissue sections was reduced and was more variable from case to case compared with AMeX-fixed and paraffin-embedded tissue sections (Fig. 6A, B), and no association with the levels of nm23 protein by immunoblotting was observed.

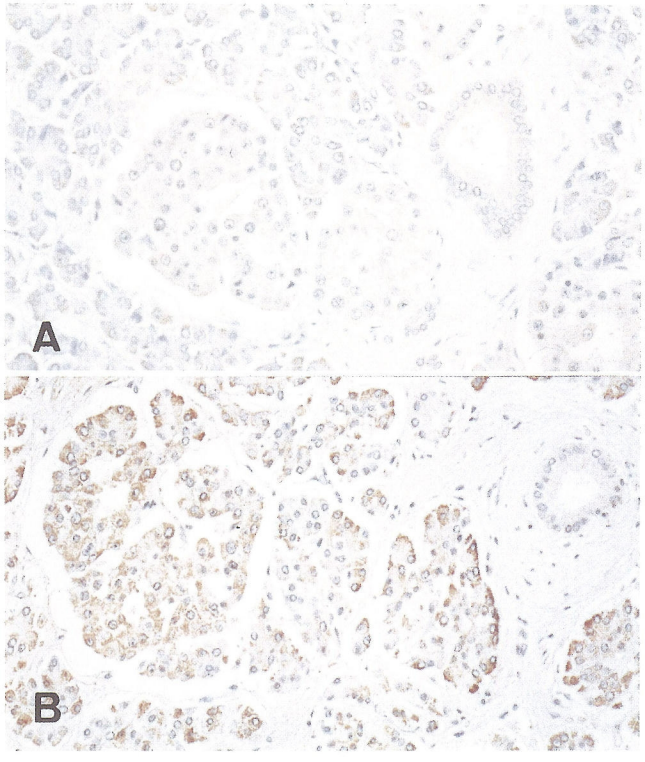

Fig. 6. The difference in stainability of nm23 protein detected by Pan-242 in both formalin-fixed paraffin-embedded and AMeX-fixed paraffin-embedded pancreatic tissues obtained from the same case. In the formalin-fixed paraffin-embedded tissue (**A**), staining intensity was markedly reduced and its pattern was consistently less clear compared with that in AMeX-fixed paraffin-embedded tissue (**B**). ×200.

IV. Discussion

Densitometry has frequently been used for quantitative analysis of Southern, Northern and dot blotting [24], but less so for the quantitative analysis of proteins. Recently, a non-isotopic blotting method using chemiluminescent detection has been developed [12] and was applied to semiquantitative analysis of immunoblotting by Huang and Amero [13]. Ayhan *et al.* showed nm23 protein expression in colorectal carcinomas by this method, but the details of the quantification were not described [2]. Our present data confirmed that the range in which the densitometric value correlates with the protein level was limited, and whenever the levels of protein were out of measurement range, a quantitative analysis was difficult. To perform a semiquantitative analysis by densitometry of immunoblotting signals, the precise representation of the original protein levels is a prerequisite. The usefulness and the limitation of semiquantitative immunoblotting method using the chemiluminescence detection system was also reported by Huang and Amero [13]. Ayhan *et al.* showed nm23 protein expression in colorectal carcinomas by this method, but the details of the quantification were not described [2]. Our present data confirmed that the range in which the densitometric value correlates with protein level was limited, and whenever the levels of protein were out of measurement range, quantitative analysis was difficult. To perform a semiquantitative analysis by densitometry of immunoblotting signals, the precise representation of the original protein levels is a prerequisite. The usefulness and the limitation of semiquantitative immunoblotting method using the chemiluminescence detection system was also reported by Huang and Amero [13]. They pointed out that the relationship between the amount of total protein and the antigen signal was specific as detected by the chemiluminescence-based immunoblotting, so it must be determined empirically. A comparison of the data should be made only within the range in which an approximately linear graph can be obtained (See Fig. 2).

The details of nm23-H1 and H2 isoform levels and their ratio in human normal or tumor tissues have not yet

Fig. 4. Relative amount of nm23 protein and the H2/H1 ratio in human normal tissues. The total amount of nm23 protein and the H2/H1 ratio varied from tissue to tissue. Liver contained the largest amount of nm23 protein, while mammary gland, heart and skeletal muscle contained low levels of nm23 protein. The level of H2 isoform was higher than that of H1 in all tissues.

Fig. 5. Immunohistochemical localization of nm23 protein on AMeX-fixed paraffin-embedded tissues detected by Pan-242. Nm23 protein was mainly localized in the cytoplasm. Tissues could be divided into three groups according to the staining intensity and pattern as follows. Homogeneous and strongly positive (**A**, liver; **B**, pancreas), homogeneous and weakly positive (**C**, heart), and heterogeneous staining pattern (**D**, adrenal gland; **E**, kidney and **F**, gray matter of brain). ×150.

been investigated. We found that two out of three pancreases showed no specific bands at 18 kD and 20.5 kD, instead presenting only lower molecular weight smears, suggesting enzymatic degradation of proteins. In such cases, nonspecific staining was also observed in immunostaining, and neither localization nor expression level showed consistent results. Nakamori *et al.* reported that nm23 protein was not detected in normal human pancreases either by immunoblotting or by immunostaining [19]. However, the present study demonstrated that two bands corresponding to H1 and H2 isoforms were detected in pancreases, and parenchymal cells including islets, acini and ducts were strongly stained homogeneously. This difference may reflect various levels of protein degradation, highlighting the need for careful handling of tissue samples in such studies.

The changes in nm23 gene expression have been studied immunohistochemically by many authors with formalin-fixed and paraffin-embedded tissue sections. In the present study, we demonstrated that staining intensity was remarkably decreased and the strength varied from case to case in 10% formalin-fixed and paraffin-embedded tissues in comparison with that of AMeX-fixed and paraffin-embedded tissues. We recommend that a precise correlation between alteration of nm23 proteins and metastatic ability of the tumor should be obtained by both immunohistochemistry and quantitative immunoblotting with AMeX-fixed and paraffin-embedded or frozen tissues. The same pattern of nm23 protein expression in endometrial carcinoma was reported by Watanabe *et al.* using immunohistochemistry with AMeX-fixed and paraffin-embedded tissues and immunoblotting with frozen tissues [34].

Recently, many investigators have provided evidence that nm23-H1 and -H2 might have different functions. Arai *et al.* reported that the expression of nm23-H1 in papillary carcinoma of the thyroid was inversely correlated with metastasis, while no correlation was found in H2 [1]. The same results were reported in breast cancer by Tokunaga *et al.* [30] and in prostate carcinoma by Konishi *et al* [16]. Okabe-Kado *et al.* showed that nm23-H2 had cytokine-like activity as a differentiation inhibiting factor [14], and Postel *et al.* showed that nm23-H2 might play a role as a c-myc transcription factor [22]. However, in earlier reports many investigators studied the relationship between the expression of nm23 protein and cancer metastasis by immunostaining using the antibodies which recognize a common epitope of nm23-H1 and -H2 protein [3, 4, 10, 11, 19, 20]. As we demonstrated that nm23-H2 levels were always higher than H1 levels in every tissue by immunoblotting, the amount of H2 protein may contribute more to the staining results in these studies. Therefore, to investigate the expression of a given gene whose product consists of several isoforms with different functions, one should examine the individual expression of each isoform.

In conclusion, we report here the various expression level of nm23 protein and its H2/H1 ratio in human normal tissues. These findings suggest that, in the assessment of changes of nm23 expression in cancer, it is important to examine the changes of each isoform level and their ratio based upon the expression levels in their corresponding normal counterparts. Further investigation with such a strategy will likely elucidate the role of nm23 as a prognostic factor in cancer metastasis.

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