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Short Communication

NM23 Protein in Neoplastic and Nonneoplastic Thyroid Tissues

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The expression of nm23 gene products has been associated with a lower metastatic potential and better outcome in malignant tumors. We have used immunohistochemistry to study the expression of nm23 protein in thyroid tissues from 101 patients consisting of 78 malignant neoplasms, 13 adenomas, and 10 other benign conditions. Cytoplasmic staining for nm23 protein was identified in normal tissues and in most benign and malignant lesions and did not correlate with either histological type or clinical outcome. Nuclear staining was seen in 93% of normal tissues and in 29% of primary carcinomas of the thyroid and was associated with a longer disease-free survival (P = 0.03). Membranous staining was present in some tumors but absent in normal thyroid. In conclusion, nm23 protein has a combined pattern of distribution among subcellular compartments in thyroid tissues. Although there was no significant association between cytoplasmic or membranous expression and histological type of tumor or survival, nm23 nuclear expression may be a useful marker in assessing the evolution of thyroid tumors. (Am J Pathol 1994, 145:26–32)

The nm23 gene was first identified in rodents on the basis of its lower expression in highly metastatic melanoma cell lines, as opposed to related low or nonmetastatic melanoma cell lines.[1] In human tissues two nm23 genes were identified, nm23-H1[2] and nm23-H2.[3] Expression of nm23 at the RNA or protein levels has been inversely correlated with the development of metastases or poor patient clinical course in cohorts of several human tumor types. These include breast carcinoma,[4–10] hepatocellular carcinoma,[11] melanoma,[12] and gastric carcinoma.[13] In other tissue cohorts, however, expression levels of nm23 failed to be inversely correlated with metastatic progression, including childhood neuroblastoma, colorectal carcinoma, and lung carcinoma.[14–16] Although the data could be interpreted to conclude that nm23 was irrelevant to metastatic progression in these tissue types, an alternative hypothesis proposed that nm23 was "inactivated" by means other than its simple reduced expression. Data that support the latter hypothesis include the identification of nm23 mutations in metastatic tumors from colorectal carcinoma[17] and neuroblastoma.[18] Evidence for a functional involvement of nm23 expression in determining tumor metastatic potential was published for the murine nm23-H1 cDNA transfected into a murine melanoma cell line[19] and for the nm23-H1 cDNA transfected into a human breast carcinoma cell line.[18] In both cases, overexpression of nm23 resulted in no significant differences in primary tumor size but caused significant reductions in metastatic potential and altered cytokine responsiveness in colonization and motility assays.[20]

The mechanism of nm23 participation in metastatic dissemination is unknown. At least five biochemical properties have been proposed for the encoded nm23 proteins that could potentially be important to its regulatory effects: First, nm23 proteins exhibit nucleoside diphosphate kinase (NDPK) activity, which may modulate nucleotide pool levels, activation of G proteins, and microtubule polymerization. However, in control and nm23-transfected cell lines,

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NDPK activity failed to correlate with nm23 expression levels and metastatic suppression, suggesting that this activity is not directly responsible for nm23 regulatory effects.[21] Second, secretion of nm23 protein was reported in myeloid cells,[22] however, we failed to detect secreted nm23 protein in breast or melanoma cell lines.[23] Third, in a *Drosophila* model system, nm23 was associated with a protein exhibiting some homology to GAP proteins.[24] Fourth, nm23 has been proposed to exhibit transcriptional regulatory activity[25] and contains sequences similar to a leucine zipper.[3] Fifth, we have recently identified a reversible serine phosphorylation of nm23 protein that is thermodynamically distinct from the phosphorylation involved in NDPK activity.[26] Levels of nm23 phosphoserine were directly correlated with nm23 expression among melanoma cell lines. The nm23 protein has been identified in the cytoplasm and the nucleus of cells[2] and recently on the cell surface.[27] In addition to its biochemical activity, it is also unknown in which subcellular compartment nm23 exerts a regulatory effect.

We have analyzed nm23 expression levels and subcellular distribution in thyroid tissues. The spectrum of thyroid pathology ranges from benign nonneoplastic conditions to highly malignant tumors. However, most of the patients undergoing thyroid surgery will have a diagnosis of a benign condition such as nodular goiter or thyroid adenoma. Among thyroid malignancies, approximately 80% are represented by papillary carcinoma,[28] which is known to have a very good prognosis. However, some histological subtypes of papillary carcinoma, such as the tall cell variant and other nonpapillary malignancies, do not share that favorable outcome and may recur or produce metastases in a short period of time.[28] The nm23 expression has been controversial in thyroid tissues. Reduced expression of nm23-H1 but not nm23-H2 RNA levels was reported in papillary tumors from patients with lymph node metastases by Arai et al.[29] However, using a monoclonal antibody to nm23-H1, Luo et al[30] failed to detect these differences at the protein level.

In this report we have analyzed thyroid tissues from 101 patients using an affinity-purified antibody that recognizes both nm23-H1 and nm23-H2. We also failed to observe a correlation between nm23 cytoplasmic expression levels and metastatic progression. However, for the first time, we observed differences in subcellular nm23 distribution. These changes in subcellular distribution may permit the development of additional hypotheses concerning the mechanism of nm23 regulatory action.

Materials and Methods

Patients and Tissues

Thyroid tissue samples from 101 patients were obtained from the files of the Surgical Pathology Section of the Laboratory of Pathology, NCI (Bethesda, MD) and from personal consultation files (MJM). Tissues from primary malignant thyroid tumors (58) and/or lymph node metastases and/or distant metastases were examined in 78 patients and were diagnosed as papillary carcinoma (35), papillary carcinoma tall cell variant (PCTC) (19), follicular carcinomas (16), medullary carcinoma (7), and undifferentiated carcinoma (1).

Thirteen patients had benign lesions represented by 10 follicular adenomas and 3 atypical follicular adenomas. Ten patients had benign nontumorous thyroid conditions (2 normal thyroids, 2 chronic nonspecific thyroiditis, 2 Graves' disease, and 4 Hashimoto's thyroiditis). For each case, a minimum of three slides were examined when available.

Most of the patients with malignant neoplasms subsequently underwent total thyroidectomy and cervical lymph node dissection followed by therapy with ^{131}I. In a few instances, surgery consisted only of lobectomy and lymph node dissection. The 58 patients with primary malignant tumors studied immunohistochemically had a mean age of 43 (range, 17 to 80 yr). The mean follow-up for these patients was 43.3 months (range, 1 to 384 months). Only one patient died of his disease (Hurthle cell carcinoma) 59 months after diagnosis. Eight patients developed distant metastases (3 patients at the time of diagnosis and the other 5 patients from 16 months to 32 years after diagnosis). Three other patients developed recurrence within 3 years after diagnosis.

Antibody

The primary antibody used for immunohistochemistry was an affinity-purified anti-nm23 peptide 11 antibody recognizing both nm23-H1 and nm23-H2 proteins.[2] This antibody was prepared in the Laboratory of Pathology, NCI (PSS).

Immunohistochemistry

Tissues were routinely fixed in 10% buffered formalin and embedded in paraffin. The slides were dewaxed in xylene and then rehydrated. After 10 minutes incubation in 0.5% H_2O_2 absolute methanol and washing in 0.01 mol/L phosphate-buffered saline (PBS),

pH 7.5, the slides were incubated with 2% normal goat serum-PBS for 20 minutes. The slides were next blotted before overnight incubation with the primary antipeptide 11 antibody diluted 1:50 in PBS. The next day, the slides were washed three times in PBS then incubated with biotinylated goat anti-rabbit immunoglobulin G and the avidin-biotin-peroxidase complex, according to recommendations of the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The sections were then incubated 3 minutes with 0.03% 3-3′ diaminobenzidine tetrahydrochloride (Sigma Chemicals, St. Louis, MO) and 0.2% H_2O_2 in 0.05 mol/L Tris buffer, pH 7.4. After rinsing in deionized water and counterstaining in hematoxylin, the slides were dehydrated and mounted.

The specificity of the staining was demonstrated with some negative control (ie, without primary antibody and with 24 hours preincubation of the primary antibody with peptide 11). Preincubation with another unrelated peptide did not make the staining disappear.

The cytoplasmic, nuclear, and membranous immunohistochemical staining were analyzed separately. For each of those criteria, the staining was considered negative when no cell was stained on the section. When a few cells or more were positive, the case was called positive for the criterion considered.

Statistical Analysis

Statistical analysis was performed for each of the above criteria (cytoplasmic, nuclear, and membranous staining) using χ^2 square test or Fisher exact test. Disease-free survival curves were determined using the Kaplan-Meier method[31] and the probability of difference between curves was tested with the Mantel-Haenszel procedure (log-rank test).[32]

Results

In agreement with previous reports,[2] staining was observed in the cytoplasmic and nuclear compartments of cells, often with combined patterns. Cytoplasmic and nuclear staining were always homogeneous never granular (Figure 1, A). Occasional membranous staining was also observed (Figure 1, B). All results are summarized in Table 1.

Benign Nonneoplastic Thyroid

The distribution of nm23 protein in normal thyroid was evaluated in 2 normal cases and in 39 cases of malignant tumor in which adjacent and distant nontumorous tissue was available (subgroup in Table 1). Some of those cases showed mild nonspecific thyroiditis or focal lesions with changes consistent with palpation thyroiditis. The staining of normal follicular epithelial cells was cytoplasmic and nuclear in 35 of 41 (85%) cases and the nuclear staining itself was virtually always present, at least in a few cells (38 of 41 (93%) normal cases. No membranous staining was seen. The intensity of the immunoreactivity was variable. The colloid stained in some cases, as well as nonepithelial cells such as endothelial cells and plasma cells.

The four Hashimoto's thyroiditis cases were characterized histologically by marked lymphoplasmocytic infiltration with formation of germinal centers. Thyroid follicles were often atrophic and oxyphilic metaplastic epithelial cells were prominent. All cases showed a strong and diffuse cytoplasmic staining in oxyphilic cells with no stained nuclei. Some lymphoid cells also stained. The two cases of Graves' disease showed some lymphocytic infiltrates with moderate hyperplasia of the follicular epithelium. The staining of

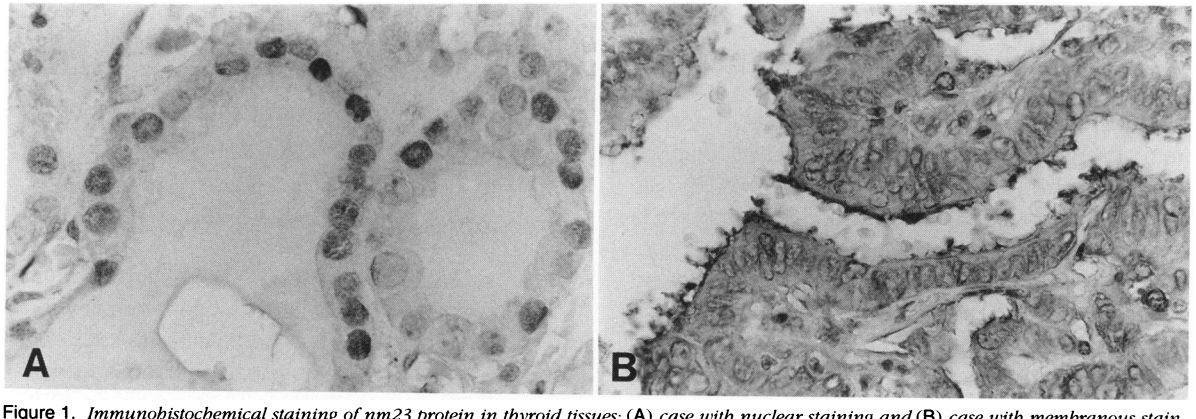

Figure 1. *Immunohistochemical staining of nm23 protein in thyroid tissues:* (**A**) *case with nuclear staining and* (**B**) *case with membranous staining.* **A:** *Nuclear staining (immunoperoxidase ×250).* **B:** *Membranous staining (immunoperoxidase ×250).*

Table 1. *Distribution of nm23 protein immunohistochemical staining in 101 specimens of thyroid lesions.*

	Staining				
	Cytoplasm	Nucleus	Membrane	Total*	Comparisons
Nonneoplastic specimen	10 (100%)	5 (50%)	0 (0%)	10	
Hashimotos'	4 (100%)	0 (0%)	0 (0%)	4	
Graves'	2 (100%)	1 (50%)	0 (0%)	2	
NST	2 (100%)	2 (100%)	0 (0%)	2	
Normal	2 (100%)	2 (100%)	0 (0%)	2	
Benign tumors	13 (100%)	6 (46%)	2 (15%)	13	
FA	10 (100%)	6 (60%)	2 (20%)	10	
AFA	3 (100%)	0 (0%)	0 (0%)	3	P (N) = 0.04
Carcinoma thyroid	52 (90%)	17 (29%)	9 (16%)	58	
PCNTC	23 (88%)	10 (38%)	4 (15%)	26	P (N) = 0.06
PCTC	13 (87%)	1 (7%)	2 (13%)	15	
FC	10 (91%)	5 (45%)	2 (18%)	11	P (N) = 0.05
MC	6 (100%)	1 (17%)	1 (17%)	6	
NTCM	39 (91%)	16 (37%)	7 (16%)	43	
Cervical lymph nodes	13 (87%)	4 (27%)	4 (27%)	15	
Neck recurrences	6 (86%)	1 (14%)	2 (29%)	7	
Distant metastases	6 (86%)	4 (57%)	4 (57%)	7	
Normal/malignant†					
ANT	33 (85%)	36 (92%)	0 (0%)	39	P (N) = 0.0001
Carcinoma	33 (85%)	12 (31%)	6 (15%)	39	P (M) = 0.03

Each cell compartment is evaluated (cytoplasm, nucleus, and membrane). PCNTC, papillary carcinoma nontall cell; PCTC, papillary carcinoma tall cell variant; FC, follicular carcinoma; MC, medullary carcinoma; NTCM, nontall cell malignancies (=PCNTC + FC + MC); FA, follicular adenoma; AFA, atypical follicular adenoma; NST, nonspecific thyroiditis; P (N) = P value for nuclear staining; P (M) = P value for membranous staining.

* Several specimens were studied for some patients.

† Subgroup of 39 cases where malignant tissue and adjacent normal tissue (ANT) were both studied.

Graves' disease and of nonspecific thyroiditis were comparable to normal thyroid tissue.

Benign Thyroid Tumors

All 13 benign thyroid tumors had moderate cytoplasmic staining in the majority of cells and 2 follicular adenomas had moderate membranous staining. There was no nuclear staining in 7 of 13 cases (54%) cases and particularly in 3 of 3 atypical adenomas.

Primary Malignant Thyroid Tumors

Fifty-eight samples from primary malignant thyroid tumors were examined. Only three cases had no staining at all. A large majority (52 of 58, 90%) exhibited a cytoplasmic staining on most cells and 17 of 58 (29%) cases had nuclear staining. In 9 of 58 (16%) cases a mild to moderate membranous staining was observed in some cells. The comparison between malignant and nontumoral thyroid was made in the 39 cases in which both malignant and adjacent normal tissue were available. Nuclear staining was much more frequent in nonneoplastic tissues (36 of 39, 92%) than in malignant tissue (12 of 39, 31%) (P = 0.0001). Membranous staining was more often present in malignant tissue (6 of 39, 15%) than in nonneoplastic tissue (0 of 39) (P = 0.03). According to our data, the group of benign tumors was not statistically different from primary malignant tumors for the expression of nm23 protein.

The 58 primary malignant tumors have been subdivided into histological subtypes (Table 1). Papillary carcinomas nontall cells variant (PCNTC) (26 cases) and follicular carcinomas (11 cases) and medullary carcinomas (6 cases) did not show any statistically significant difference for cytoplasmic, nuclear, or membranous staining when compared with the entire group of 58 tumors. In contrast, the group of PCTC had a particular pattern of staining, with only 1 of these 15 (7%) cases showing nuclear staining. In other words, PCTC lack nuclear staining more often than other thyroid malignancies or benign tissues. No difference was shown for cytoplasmic and membranous staining.

Cytoplasmic and membranous staining for nm23 protein did not appear to be correlated with patient outcome. Conversely, there was a longer disease-free survival in patients whose tumors had positive nuclear staining (P = 0.03) compared with the tumors without nuclear staining (Figure 2). Too few cases of PCTC had a follow-up to allow statistical conclusions.

Recurrent and Metastatic Tumors

The group of recurrent tumors (7 cases) and the group of lymph node metastases (15 cases) and distant metastases (7 cases) did not exhibit a statistically

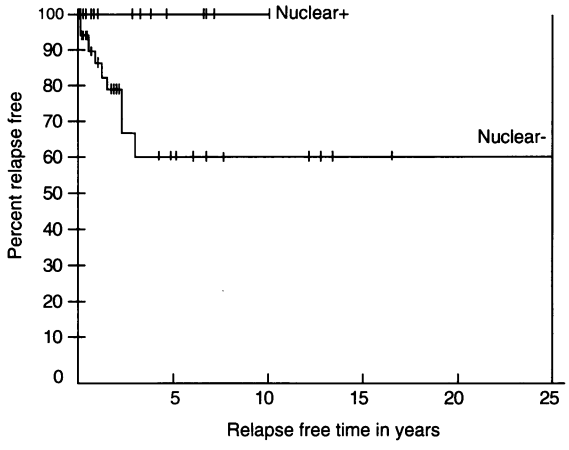

Figure 2. *Relationship between nm23 nuclear expression and disease-free survival in 58 patients with primary thyroid tumors (nuclear+, 16 tumors with stained nuclei; nuclear–, 42 tumors without stained nuclei).*

significant difference in staining pattern when compared with primary thyroid tumors. Although not statistically significant, membranous staining increased from 16% (range, 13 to 18% by histological type) of primary tumors to 36% (range, 27 to 57%) of metastatic tumors. There were no significant differences in staining between primary tumors and lymph node metastases among nine cases of paired samples.

Discussion

This report has examined nm23 protein expression levels and subcellular distribution among thyroid tissues from 101 patients. The prognostic potential of nm23 expression has been controversial in the thyroid, with one report indicating reduced nm23-H1 expression[29] and another report finding no significant trend with lymph node metastasis.[30] Although nm23 protein has been reported in the cytoplasmic and nuclear compartments of tumor cells[2] and on the cell surface,[27] to date, immunohistochemical analyses in virtually all cancer types have focused solely on cytoplasmic staining. When we quantitated cytoplasmic staining intensity, variability was observed in all of the histological types of thyroid tissues with no significant trends, in agreement with the report of Luo et al.[30]

Our data suggest the first association of nm23 subcellular distribution and disease progression. Nuclear staining was observed in 93% of normal tissues and declined to 46% of benign tumors, 29% of primary carcinomas, and 31% of recurrent or metastatic lesions. Consistent with the relative overexpression of nuclear nm23 staining in normal tissues, nuclear staining within a subpopulation of primary thyroid carcinomas predicted longer patient disease-free survival. On the contrary, membranous nm23 staining was not observable in normal tissues, rose to 15% of benign tumors and 16% of primary carcinomas and then rose again to 33% of metastatic lesions. Statistical significance was not attained in this trend, possibly due to a small sample size in the metastatic lesions. Progression in thyroid disease was therefore accompanied by a general decrease in nuclear staining and an increase in membranous staining. Whether this trend occurs in other cancer types and its relationship to decreased cytoplasmic nm23 staining observed in aggressive breast, hepatocellular and gastric carcinomas, and melanoma remains to be determined. However, the data suggest the hypothesis that dysregulation of the distribution of nm23 protein may also be an important determinant of its regulatory activity.

The role of nm23 protein in the cell nucleus is open to speculation. The nm23 proteins were hypothesized to contain a leucine zipper motif that is common to transcription factors,[3] although X-ray diffraction studies of a slime mold homologue indicated that the orientation of the leucines along this motif was incompatible with this proposed function.[33] Recently, nm23 was reported to be a transcription factor regulating a Puf site found in the c-*myc* and other promoters.[25] Other roles for nm23 in the nucleus, such as chaperone proteins and nuclear matrix components, cannot be eliminated as yet.

The finding reported herein that a proportion of nm23 protein may be localized to the cell membrane also raises questions as to what biochemical function this protein may be exhibiting in this location. Transfection data in murine melanoma cells and human breast carcinoma cells indicate that overexpression of nm23 inhibits tumor cell responsiveness to a variety of cytokines, ie, transforming growth factor-β in colonization of insulin-like growth factor and platelet-derived growth factor in motility.[18–20] These data suggest an interaction of nm23 and proteins involved in the signal transduction process, possibly at the cell membrane level. The nm23 protein has been colocalized to G proteins in the membrane, although its role as an NDPK in G protein activation has been debated.[23] Additional functions at or near the cell membrane await further experimentation.

Combined patterns of staining have already been observed in other tissues. Cytoplasmic staining of nm23 protein has been noted in breast,[2,4,34,35] lung,[16] and liver.[11] Membranous and cytoplasmic staining have been reported by Sastre-Garau et al[35] in breast carcinoma. Nuclear staining in association with cytoplasmic staining was also described by

Rosengard et al[2] in breast carcinoma using Western blots and immunohistochemistry.

The distribution of nm23-H1 products in normal breast is almost always cytoplasmic,[4,6] consistent with our data in normal thyroid in which only 6 of 39 cases had no cytoplasmic staining. Differences of expression in malignant tumors and in corresponding normal tissue have already been reported in breast[34,35] and in colon.[15] In those cases, the difference consisted of a stronger cytoplasmic staining in malignant cells.

Our results based on the entire group of primary malignant thyroid tumors suggest that the expression of nm23 in the nuclei of tumoral cells may be associated with a longer disease-free survival. Comparison of the two disease-free survival curves between two groups with or without nuclear staining shows earlier recurrences or metastases in the latter group. In our study, the lack of nuclear staining in most PCTC might also have a prognostic implication, because this kind of tumor, first described by Hawk and Hazard,[36] makes up approximately 10% of the papillary cancers and is indeed known to behave more aggressively than PCNTC.[36,37] In our series, several patients with PCTC were lost to follow-up and we could not confirm that data. Follicular carcinomas and medullary carcinomas did not show any specific pattern of staining, although they are well known to have a worse prognosis than papillary carcinomas. Samples from metastatic sites or from primary tumors with metastatic extension at the time of diagnosis showed an increased frequency of membranous staining. However, the size of these subgroups was too small to allow statistical conclusions.

It follows from our study that in thyroid tissues the nm23-H1 protein has a combined pattern of distribution and that its expression may be a useful marker in assessing the evolution of thyroid tumors. However, this association should be confirmed by larger series of thyroid carcinomas with longer patient follow-up data.

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