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Expression of *nm*23-H1 and *nm*23-H2 Proteins in Prostate Carcinoma

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The *nm*23 gene products/nucleoside diphosphate (NDP) kinase expression in prostate carcinomas and benign hyperplasias was evaluated immunohistochemically. Monoclonal antibodies against *nm*23-H1 and *nm*23-H2 proteins were prepared using the corresponding proteins fused with glutathione S-transferase as immunogens. Of the 80 cases of nonmetastatic prostate carcinoma examined, 74% (59/80) and 60% (48/80) were immunoreactive for *nm*23-H1 or *nm*23-H2 protein, respectively. Negative staining for *nm*23-H1 occurred in 83% of metastatic lesions, while 34% were negative for *nm*23-H2. All primary tumors corresponding to the metastases examined showed positive immunostaining for *nm*23-H1, indicating an inverse relationship between expression of this protein and metastatic status. *nm*23-H2 protein was detected in 83% of primary tumors and its expression appeared to be significantly correlated to the degree of histological differentiation. In contrast, all cases of benign prostatic hyperplasia showed elevated levels of both *nm*23-H1 and *nm*23-H2 expression. These data suggest that the *nm*23/NDP kinase may play a role in suppressing the expression of malignant potential in prostate carcinomas.

Key words: *nm*23-H1 — *nm*23-H2 — Metastasis — Prostate carcinoma

Tumor metastasis is the result of multiple, sequential cellular events, and it remains a major cause of mortality among cancer patients. Recent studies of the genetic basis of tumor spread have implicated the *nm*23 gene as a suppressor of metastasis, as demonstrated by the reduced expression of *nm*23 proteins in certain rodent model systems.[1] Transfecting *nm*23 cDNA into otherwise highly malignant K-1735 TK murine melanoma cells significantly reduces their *in vivo* metastatic potential.[2] In some human tumors, such as breast, gastric and hepatocellular carcinomas, a strong association has been reported between reduced *nm*23 protein levels and acquisition of metastatic behavior.[3–7] High homology between *nm*23 proteins and NDP[4] kinases has subsequently been identified in a number of species.[8,9] In fact, NDP kinases A and B have been shown to be identical to 2 isotypes of human *nm*23 protein homologues, *nm*23-H1 and *nm*23-H2, respectively.[10] In addition, the deduced amino acid sequence of the *nm*23 gene has been found to share 78% homology with that of the *Drosophila* abnormal wing disc(*awd*) gene[11]; the *awd* protein also shows NDP kinase activity.[12]

Recent gradual increases in the incidence of prostate cancer in Japan also reflect an increase in the number of metastatic cases.[13] In an effort to determine if *nm*23 gene products are involved in prostate cancers with metastatic potential, we investigated the relative cellular levels of expression of *nm*23-H1 and *nm*23-H2 using immunohistochemical methods.

MATERIALS AND METHODS

Tissue samples Human prostate tissues were obtained from needle biopsies, transurethral resections and total prostatectomies performed at Sumitomo Hospital and Nishinomiya Prefectural Hospital. Eighty (80) cases of prostate carcinoma were analyzed from patients 60 to 90 years of age (mean 74 years) along with 12 latent carcinomas found at autopsy from men 53 to 89 years of age (mean 72 years). An additional 6 cases of primary carcinoma with concomitant metastases were examined from men aged 64 to 80 years (mean 70 years). All samples were fixed in 10% neutral buffered formalin and embedded in paraffin. They were then sectioned at 4 μm with the first section routinely stained with hematoxylin and eosin for histologic diagnosis and additional sequential sections left unstained for immunohistochemical reactions. Prostate carcinomas were classified as well, moderately or poorly differentiated adenocarcinomas according to the General Rules for Clinical and Pathological Studies on Prostatic Cancer, Japan.

Immunohistochemistry Immunostaining of tissue samples was achieved using mouse mAb[4] raised specifically against *nm*23-H1 and *nm*23-H2 proteins. The entire gene-coding regions of *nm*23-H1 and *nm*23-H2 were fused with a 26 kDa GST and inserted into *E. coli*.[9] The partly soluble fusion proteins were purified by affinity

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[4] Abbreviations: NDP, nucleoside diphosphate; mAb, monoclonal antibody; GST, glutathione S-transferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ABC, avidin-biotin-peroxide complex.

chromatography using glutathione Sepharose 4B and were subsequently shown to migrate as a single band corresponding to 46 kDa by SDS-PAGE. Mice were immunized 3 times at 2-week intervals with the purified *nm*23/GST fusion proteins. Spleen cells from the immunized animals were hybridized with murine myeloma cell line NS-1 and the resultant culture supernatants were assayed for specific *nm*23 protein reactivity using ELISA and immunoblotting. Monoclonality was attained by 3 limiting dilutions of positive cultures.

Two mAbs, H1-229 and H2-439, specific for *nm*23-H1 and *nm*23-H2, respectively, were obtained. Immunoblotting was performed to confirm the specificity using these mAbs and mouse myeloma line NS-1 transfected with either the *nm*23-H1 or H2 gene. Briefly, the proteins separated on 12.5% SDS-PAGE were electrophoretically transferred onto a nitrocellulose membrane (Sartorius) with the mAbs. Specific bands were detected using the ABC technique (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA). Immunohistochemically, the unstained tissue sections were probed for *nm*23 reactivity using the same ABC technique. Briefly, slides were deparaffinized and incubated for 30 min in 0.3% hydrogen peroxide in methanol, rinsed with phosphate-buffered saline and incubated for 20 min with dilute normal rabbit serum. Sections were then incubated again overnight at 4°C with H1-229 or H2-439 at a concentration of 1 μg/ml. The next day, tissue sections were exposed to biotin-labeled horse anti-mouse IgG for 30 min, followed by the ABC for an additional 40 min. Sections were stained with the chromogen 3,3′-diaminobenzidine tetrahydrochloride in 0.01% hydrogen peroxide and counterstained with hematoxylin for microscopic evaluation. NS-H1-9 and NS-H2-1 cells, transfected with the *nm*23-H1 or H2 gene, respectively, were fixed in cold acetone and used as positive controls. The intensity of immunostaining was categorized as strongly positive (++), moderately to weakly positive (+), or negative (−). Statistical analyses were carried out by using Fisher's exact test. Results were considered significant when the *P* value was less than 0.05.

RESULTS

Characterization of mAbs Of the mAbs which were generated, two cross-reacted with both the *nm*23-H1 and *nm*23-H2 proteins, as demonstrated by the presence of two bands in SDS-PAGE experiments. This result was not unexpected, since the NDP kinase of human erythrocytes is a hexameric enzyme with random association of the two polypeptides.[10] In contrast, mAb H1-229 and H2-439 appeared to be monospecific for *nm*23-H1 and *nm*23-H2 proteins, respectively, as demonstrated by Western blotting (Fig. 1).

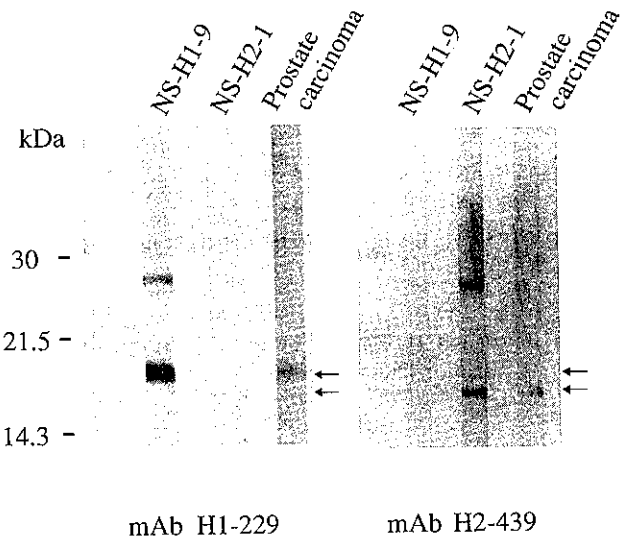

Fig. 1. Immunoblotting analysis identified 20.5 and 18 kDa proteins with mAb H1-229 or H2-439, respectively. NS-H1-9 or NS-H2-1 myeloma cells were transfected with either the *nm*23-H1 or H2 gene. Prostate carcinoma used was positive by immunohistochemistry.

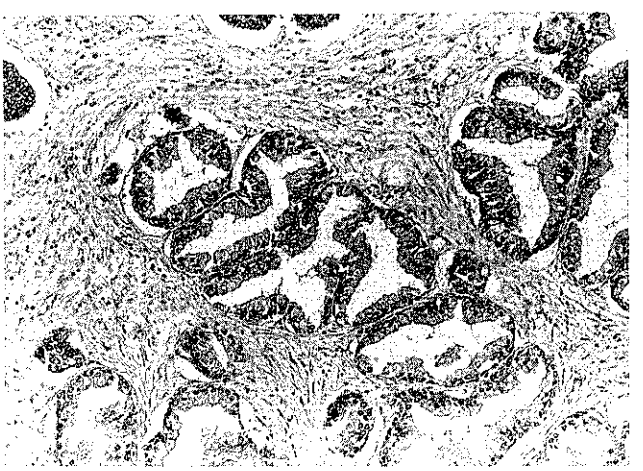

Fig. 2. Positive immunohistochemical staining of *nm*23-H1 in cytoplasm of benign prostate hyperplasia. (ABC method, counterstained with hematoxylin, ×100)

Immunohistochemical results The immunohistochemical detection of *nm*23 gene product expression was confined primarily to the cytoplasm of both normal and cancerous cells and showed a diffuse staining pattern (Fig. 2). The relationship between tumor progression and expression of *nm*23 proteins determined by immunostaining is summarized in Tables I and II. As can be

Table I. *nm*23-H1 and H2 Immunostaining in BPH and Latent, Clinical Prostate Carcinomas

	No. of cases	*nm*23-H1 (%)			*P* value	*nm*23-H2 (%)			*P* value
		(−)	(+)	(++)		(−)	(+)	(++)	
BPH	5	0 (0)	5 (100)	0 (0)		0 (0)	5 (100)	0 (0)	
Latent	12	0 (0)	11 (92)	1 (8)	0.004	1 (8)	10 (83)	1 (8)	0.176
Clinical	80	21 (26)	48 (60)	11 (14)		32 (40)	39 (49)	9 (11)	
Metastatic site	6	5 (83)	1 (17)	0 (0)		2 (34)	4 (67)	0 (0)	

BPH: Benign prostatic hyperplasia. (−): negative, (+): weakly positive, (++): strongly positive.

Table II. *nm*23-H1 and H2 Immunostaining in BPH and Prostate Carcinomas According to the Tumor Differentiation

		No. of cases	*nm*23-H1			*P* value	*nm*23-H2			*P* value
			(−)	(+)	(++)		(−)	(+)	(++)	
BPH		5	0	5	0		0	5	0	
Latent	Wel.	6	0	5	1	1.000	0	5	1	1.000
	Mod.	6	0	6	0		1	5	0	
Clinical	Wel.	12	4	8	0	0.243	6	3	3	0.013
	Mod.	32	5	22	5		10	16	6	
	Por.	36	12	18	6		16	20	0	

BPH: Benign prostatic hyperplasia. Wel.: Well-differentiated adenocarcinoma. Mod.: Moderately differentiated adenocarcinoma. Por.: Poorly differentiated adenocarcinoma.

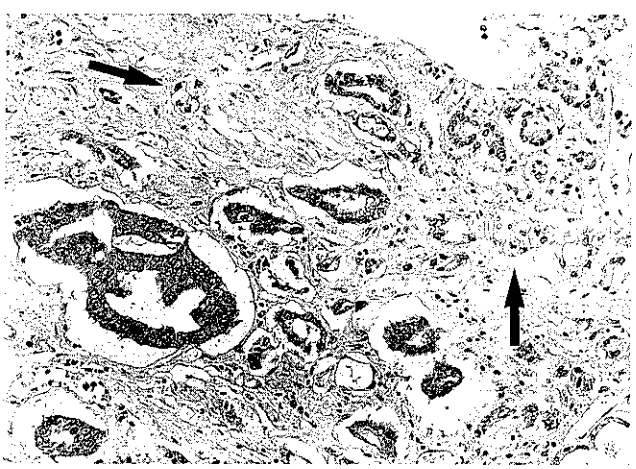

Fig. 3. *nm*23-H2 protein expression in prostate carcinoma, showing strong expression in well- to moderately differentiated adenocarcinoma, but not in the invasive edges of the tumor (arrow). (ABC method, counterstained with hematoxylin, ×100)

seen, BPH consistently exhibited only weak reactivity to both H1 and H2 proteins. Table I indicates little apparent relation between immunoreactivity of *nm*23-H2 and lesion type; however, there was a significant ($P=0.004$) decrease in *nm*23-H1 immunoreactivity from latent to clinical to metastatic carcinoma, and the overall expression of *nm*23-H1 protein was significantly reduced in metastatic vs. non-metastatic (clinical) cases ($P=0.025$).

Table II relates *nm*23 immunoreactivity to degree of tumor differentiation. In this instance, only the H2 protein expression in clinical carcinomas showed a significant correlation ($P=0.013$) to degree of differentiation when compared to BPH and latent disease. One case of latent carcinoma proved to be negative for protein and it is interesting to note that this particular tumor was histologically invasive. Lack of both *nm*23-H1 and *nm*23-H2 expression was often exhibited by the invasive edges of aggressive tumors and in poorly differentiated cancer cells (Fig. 3). The staining intensity of primary tumors compared to that of the corresponding metastases is summarized in Table III. The cases analyzed represent 2 moderately and 4 poorly differentiated carcinomas. Expression of *nm*23-H1 protein was significantly reduced in the metastatic lesions (Fig. 4) over the expression seen in the primary tumors ($P=0.007$), but there was no correlation between *nm*23-H2 protein staining and lesion site (primary vs. metastatic sites).

DISCUSSION

Our study shows that the expression of *nm*23-H1 protein was significantly lower in metastatic lesions than in their primary tumors. Similar reduced expression was

Table III. *nm*23-H1 and H2 Immunostaining in Primary and Metastatic Sites of Prostate Carcinoma

	No. of cases	*nm*23-H1			*P* value	*nm*23-H2			*P* value
		(−)	(+)	(++)		(−)	(+)	(++)	
Primary	6	0	6	0	0.007	1	5	0	0.500
Metastastic	6	5	1	0		2	4	0	

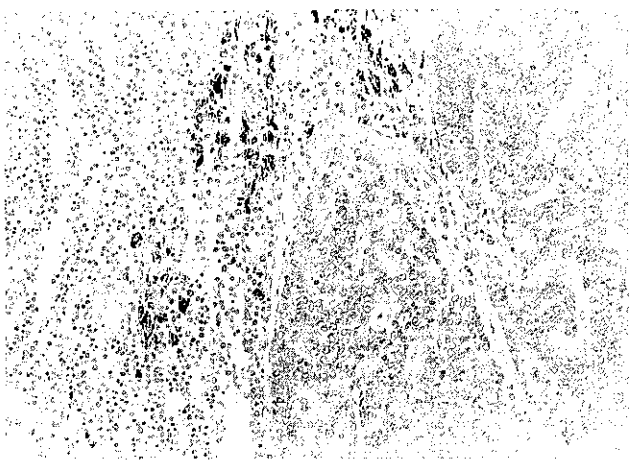

Fig. 4. Prostate carcinoma in the pulmonary lymph node was negative for *nm*23-H1 protein. (ABC method, counterstained with hematoxylin, ×100)

observed when latent, clinical, and metastatic tumors were compared. However, this tendency did not correlate statistically with the degree of histological differentiation. The expression of *nm*23-H1 has been reported to be inversely associated with metastatic potential in several human tumors,[3-7, 14] as well as in experimental models.[1, 2]

Recent studies have identified a second human *nm*23 gene, *nm*23-H2, which exhibits 88% identity to *nm*23-H1. Decreases in the level of *nm*23-H1 mRNA in human breast carcinomas have been found to be more closely associated with a high metastatic potential than decreases in the level of *nm*23-H2 mRNA.[15] This is in agreement with the results of the present study, which suggest that *nm*23-H1 staining intensity is more closely associated with metastatic behavior. The expression of *nm*23-H2 protein, however, appeared to correlate with the degree of clinical tumor differentiation, rather than with the metastatic potential. This is also indicated indirectly by the fact that invasive areas of otherwise immunoreactive tumors and poorly differentiated lesions were often negative or only weakly positive for *nm*23-H2 protein.

Similar NDP kinase activities have been attributed to both *nm*23-H1 and *nm*23-H2 proteins.[9] In cells, NDP kinase provides nucleoside triphosphates other than ATP. It has been suggested that this protein plays a role in signal transduction, supplying GTP to G-proteins, including *ras* p21,[16, 17] and is associated with the function of microtubules, which are components of the cytoskeleton and play a role in the formation of the mitotic spindle in cell division.[18] There is no evidence, as yet, for a role of NDP kinase in suppression of metastasis. However, in some tumors *nm*23 proteins are suggestively associated in neoplastic progression and malignancy. Advanced-stage colorectal carcinomas have been shown to exhibit higher levels of *nm*23 mRNA than normal mucosa,[19] and neuroblastomas in advanced stages demonstrate increased levels of *nm*23-H1 protein relative to early lesions.[20] It is possible that any metastatic enhancer/suppressor functions of *nm*23 are tumor-specific, and/or that regulatory mechanisms attributable to *nm*23 vary with intra- and extracellular biochemical conditions. To date, a role in induction or augmentation of metastatic propensity in transfection assays has been demonstrated for 10 oncogenes.[21] But many other genes, including suppressor genes which may have themselves been inactivated or mutated, are likely to be involved in the control of tumor progression, and particularly in metastasis. For instance, in prostate cancer, increased aggressiveness and metastatic growth are demonstrable phenotypic expressions of *ras* oncogene activation,[13, 22, 23] while another recent report describes reduced levels of retinoblastoma gene protein in prostate metastases as detected by immunoperoxidase histochemistry.[24]

In conclusion, the present study suggests a role for the *nm*23 gene products in prostate carcinoma progression, in that their reduced expression is correlated to increased malignant potential. Reduced expression of *nm*23-H1 protein appears to be associated with metastatic tendency, while *nm*23-H2 expression may be associated with degree of tumor differentiation.

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